THE JOURNAL OF NUTRITION

VOLUME 30

JULY – DECEMBER, 1945

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

CONTENTS

No. 1 JULY, 1945

No. 2 AUGUST, 1945

No. 3 SEPTEMBER, 1945

No. 4 OCTOBER, 1945

No. 5 NOVEMBER, 1945

No. 6 DECEMBER, 1945

THE NUTRITIONAL VALUE OF TELANG LIVERS [1]

PAUL L. PAVCEK, EDWARD J HERBST AND C A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

(Received for publication January 24, 1945)

Telangiectasis in cattle livers has been noted since 1900 and has been the subject of various reports (Schote, '36, and Carta, '38). The abnormality is characterized by dark red or purplish depressed areas beneath the capsule of the liver which vary in size from a few millimeters to 15 mm. in diameter. On gross sections, the dark areas can be seen distributed through the entire liver, although in some cases only one lobe may be involved. The dark areas are soft and spongy and are usually engorged with blood.

It has been reported by Smith ('40) that nearly 3% of all beef livers inspected each year in the United States are condemned for telangiectasis. In the meat packing industry the common name of these livers is telang livers. Only a few suggestions as to the possible pathogenesis of the condition are available. Experiments at Kansas State College ('40) disclosed a high incidence of telang lesions in the livers from cattle fed high fat rations. Frederick ('43) pointed out that the incidence of telangiectasis tends to be greatest in steers which have been on highly concentrated fattening rations for extended periods of time.

The experiments to be reported in this paper were undertaken with the aim of establishing the nutritional value of these livers and possibly to cast some light on the pathogenesis of the condition. Feeding tests and certain chemical and vitamin analyses were made on representative samples of both normal and telang livers.

MINERAL AND PROXIMATE COMPOSITION

Twenty-six livers exhibiting severe telang lesions and thirty-three normal livers were selected for the analytical work. The fat content was determined by ethyl ether extraction of dried samples from each liver. The telang livers showed a range of 19.7 to 27.6% while the

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board

normal livers contained from 15.0 to 21.3% of fat. No significance is attached to these variations in fat content. The iron content of the telang livers ranged from 27.5 to 35.0 mg. and the normal livers from 21.0 to 24.0 mg. per 100 gm. of dry matter. The copper values for the telang livers showed a wider variation from 2.0 to 13.8 mg. per 100 gm. dry matter and were somewhat lower than those for the normal liver 7.0 to 16.0 mg. per 100 gm. The higher iron content of the telang livers may have been due to the blood held in the telangiectatic lesions.

VITAMIN CONTENT OF LIVERS

Carotene and vitamin A. In the same series of twenty-six telang livers, carotene values ranged from 0.2 to 2.15 mg. per 100 gm. of fresh material. The normal livers showed a comparable spread of 0.25 to 2.0 mg. per 100 gm. Of twenty-six telang livers assayed for preformed vitamin A (Davies, '33), eighteen of them had vitamin A values of 10 to 60 mg. per 100 gm. In contrast to this, only one of the thirty-three normal samples had an especially high content, 55 mg., and the majority of the samples contained less than 10 mg. per 100 gm. of fresh liver.

Ascorbic acid. Vitamin C determinations by the indophenol technique (Mindlin and Butler, '38) indicated that both normal and telang livers contained about the same concentration, i.e., 15 to 30 mg. per 100 gm. of fresh tissue.

Choline. Determination of this substance in the dried livers was made colorimetrically after precipitation of the choline as the reineckate. Values for both types of liver were in the narrow range of 1500 to 1600 mg. per 100 gm. of dried material.

Pantothenic acid. The microbiological method for assay showed telang and normal livers to contain from 14 to 18 mg. per 100 gm. on the dry weight basis.

Niacin. Microbiological assay for this factor demonstrated that both types of liver contained between 40 and 50 mg. per 100 gm. of dried liver.

Biotin. Assays for this compound were also made microbiologically. The one sample of dried telang liver tested had a biotin content of 0.34 mg. while a normal liver assayed at the same time contained 0.63 mg. per 100 gm. It is doubtful whether this variation has any significance.

FEEDING EXPERIMENTS WITH FRESH, NORMAL AND TELANG LIVER

Preliminary work on the selection of a suitable laboratory animal to study the growth promoting effect and possibly toxicity of telang liver as compared to normal beef liver, indicated that the rat was most de-

sirable. Dogs, cats, chicks and monkeys were also investigated as possible experimental animals.

These early studies revealed that rats could be maintained from weaning to maturity, without any deleterious effects, on rations consisting of fresh normal beef liver supplemented only with 50–100 mg. of calcium carbonate per day. However, it was observed that certain telang livers fed under identical conditions were quite toxic to weanling rats. Animals fed these livers failed to grow, developed severe paralysis of the front and hind quarters which very shortly progressed until the front legs were useless. This paralysis together with bone fractures was observed in 7 to 10 days and the animal usually died within 3 weeks unless the liver feeding was discontinued. These observations suggested that the telang liver contained a specific toxic substance, not present in normal beef liver. Preliminary results (Herbst et al., '44) indicated a relationship between the toxicity and the high vitamin A content. The following experiments were carried out to study this relationship further.

Weanling Sprague-Dawley male albino rats (40–50 gm.) were used to assay forty-eight different telang livers. The livers were obtained from the Oscar Mayer Co. of Madison and from Wilson and Co., Chicago. They were received in a frozen condition and immediately stored at 0°F. Large representative samples of each liver were ground and stored at all times under freezing conditions to prevent spoilage. The degree of telang lesion formation was recorded for each liver. If the lesions comprised about one-half the total liver area, the liver was graded "Very Extensive Lesions"; if the lesions were quite numerous but made up less than one-half of the liver area, the grade "Extensive Lesions" was applied; if the lesions were scattered and limited to only a small part of the total liver area, the liver was graded "Slightly Marked".

The ground liver was placed in the food cups in the frozen condition. The excess was washed out of the cups on the following day and fresh frozen liver was again fed. Fifty to 100 mg. of calcium carbonate were added to the liver fed each rat to correct the undesirable Ca:P ratio (about 1:16) existing in beef liver. Two drops of halibut liver oil (diluted 1:5 with corn oil) were fed to each rat per week to insure an adequate intake of vitamin D.

Seventeen normal beef livers, obtained from a local butcher shop, were assayed for toxicity under conditions identical to those that existed during the telang liver assay.

The effect of the addition of 25 and 50% of fresh normal liver to toxic telang liver was studied. Equal portions of three normal livers, that had proved non-toxic in previous feeding experiments and of three toxic telang livers were ground together and thoroughly mixed. Mixtures of the same livers containing 25% of normal liver and 75% of toxic telang liver were prepared in a similar manner.

Each of the six mixtures was fed to weanling Sprague-Dawley male rats; three control rats received the undiluted toxic telang liver. Supplements of calcium carbonate and halibut liver oil were fed and in general the conditions that existed during the assay of the livers for toxicity were maintained.

RESULTS

The results of feeding the forty-eight telang livers, three of which were graded as "Very Extensive", twenty-six as "Extensive" and nineteen as "Slightly Marked", can be summarized as follows: The livers with the extensive or very extensive lesions usually showed toxicity and in a few cases designated as "Slightly Marked" also produced the typical paralysis.

Fig. 1 Weanling rat fed a toxic telang liver plus calcium carbonate ad libitum. The typical paralysis of the legs is evident.

The extreme brittleness of the leg bones appearing in the animals fed telang liver designated as toxic was the most constant symptom observed. Fractures, often multiple, occurred usually in the tibia of the rear legs and in the radii and ulnae of the forelegs. When fractures occurred in the bones of the forelegs, the legs became twisted and the animal would crawl about on the deformed limbs. Figure 1 shows

an animal typical of those which had been on the toxic telang liver diet for 7–10 days.

Growth failure always preceded the bone fragility. If the liver feeding was continued after the onset of the symptoms the animals died, usually within a week. Respiratory involvements were evident in those animals that succumbed to telang liver toxicity. The affected animals would breath laboriously and bloody discharges were often observed around the nostrils. Paralysis of the hind legs was quite common and enuresis occurred frequently.

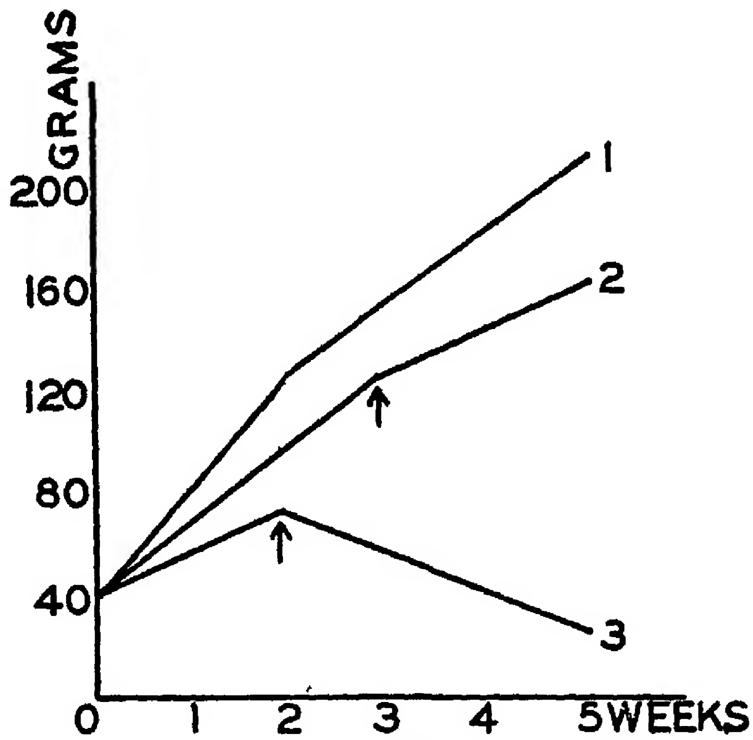


Fig. 2 Growth curves of male albino rats on fresh toxic telang-normal beef liver mixtures.
(1) Received equal portions of normal liver and toxic telang livers.
(2) Received 25% normal liver and 75% toxic telang liver.
(3) Received 100% toxic telang liver.
Arrows indicate time at which bone fractures occurred.

Post mortem examination of the animals revealed severely congested lungs indicating that death may have been due to pneumonia. The livers were unusually yellow but all other organs were normal. Since fatty livers were also observed in animals fed normal liver and non-toxic telang liver, this observation is not of great significance.

None of the seventeen normal livers assayed proved toxic to weanling rats. All animals fed calcium supplemented normal liver as the sole dietary grew extremely well and in no case were symptoms of bone fragility observed.

The results of one experiment in which mixtures of toxic telang liver and normal liver were fed to weanling rats are shown graphically in figure 2. Growth and toxicity data in two additional experiments were

quite similar to the results illustrated. Weanling rats were completely protected from the toxic effects of telang liver if their rations contained 50% of normal beef liver. The inclusion of 25% of normal liver did not afford complete protection. However, the onset of the symptoms of bone fragility was delayed and growth was considerably better than in the case of the animals fed 100% toxic liver.

VITAMIN A TOXICITY EXPERIMENTS

The literature contains frequent reports of the toxicity of large dosages of vitamin A to laboratory animals and humans. These studies have recently been reviewed by Josephs ('44).

An almost constant feature of hypervitaminosis A in rats is the development of multiple spontaneous fractures of the bones of the extremities. The close similarity of these symptoms to those observed in rats fed toxic telang liver suggested that excessively high stores of vitamin A might be present in certain of the abnormal livers.

The studies referred to above on the comparative vitamin A content which to date have involved over 100 samples of telang and normal beef liver, disclosed that the abnormal livers were consistently higher in vitamin A potency. It was impossible to establish a correlation between animal toxicity and the vitamin A storage in every liver studied since toxicity data were not available for all the samples assayed.

To illustrate the correlation which existed between the toxicity of certain samples of telang liver and their content of vitamin A, several livers of varying vitamin A content were fed to weanling rats. Total vitamin A intakes of the rats were calculated from the vitamin A potency of the liver and the amount ingested per rat per day. Data on six telang livers, two of which were toxic and four non-toxic, are summarized in table 1.

TABLE 1

Summary of vitamin A content and toxicity of telang livers

DESCRIPTION OF RATION [1]	VITAMIN A CONTENT OF LIVER	INTAKE OF VITAMIN A PER RAT PER DAY [2]	DAYS REQUIRED TO PRODUCE BONE FRACTURES	GROWTH OF RATS
	I U /100 gm	*I U*		*gm /week*
Telang liver, no. 43	121,000	22,990	7	0
Telang liver, no. 44	132,000	25,080	7	0
Telang liver, no. 45	68,400	12,996	No fractures	15
Telang liver, no. 46	50,800	9,652	No fractures	20
Telang liver, no. 47	45,200	8,588	No fractures	29
Telang liver, no 48	59,000	11,210	No fractures	26

[1] Each rat received a daily supplement of 50–100 mg. of calcium carbonate.

[2] Daily intake of vitamin A calculated from food consumption data

As further demonstration that the toxic symptoms manifested by bone fracturing were related to the high intake of vitamin A, halibut liver oil [2] and also crystalline vitamin A alcohol [3] were fed as supple-

TABLE 2

The toxicity of crystalline vitamin A and haliver oil supplements to weanling rats

DESCRIPTION OF RATION [1]	TOTAL INTAKE OF VITAMIN A/ RAT/DAY [2]	DAYS REQUIRED TO PRODUCE BONE FRACTURES			GROWTH OF RATS IN GM./WEEK		
	I.U.	Rat number			Rat number		
		1	2	3	1	2	3
1. Fresh telang liver, no. 47 plus 5 drops corn oil	8,588				31	39	13
2. Fresh telang liver, no. 47 plus 10 drops corn oil	8,588				31	33	33
3. Fresh telang liver, no. 47 plus 10,000 I.U. of crystalline vitamin A	18,588	11	14	11	8	14	2
4. Fresh telang liver, no. 47 plus 10,000 I.U. of vitamin A as haliver oil	18,588	11	11	7	5	3	17
5. Fresh telang liver, no. 47 plus 20,000 I.U. of crystalline vitamin A	28,588	7	8	3	2	−4	2
6. Fresh telang liver, no. 47 plus 20,000 I.U. of vitamin A as haliver oil	28,588	7	7	6	1	−17	−13
7. Steenbock stock ration plus 20,000 I.U. of crystalline vitamin A	20,000	11		8	3	23	3
8. Steenbock stock ration plus 20,000 I.U. of vitamin A as haliver oil	20,000		7	12	24	15	0

[1] Supplements of corn oil and vitamin A to liver and stock rations are in terms of daily intake per rat.

All animals fed liver rations received daily supplement of 50–100 mg. of calcium carbonate.

[2] Vitamin A supplied by ration plus supplement.

ments to rats in doses reported to be toxic by Josephs ('44). These supplements were fed at levels calculated to furnish 10,000 and 20,000 I.U. of vitamin A in addition to the amount furnished by the basal diet of fresh telang liver. The sample of liver chosen for the basal diet was

[2] Supplied by Abbott Laboratories, North Chicago, Ill. The oil contained 60,000 I.U. of vitamin A per gram.

[3] Obtained from Distillation Products, Inc., Rochester, N. Y.

sample number 48 which previous assay (table 1) had indicated to be non-toxic. The high vitamin A supplements were also fed in conjunction with a natural ration (Steenbock Stock Ration). Three animals in each group were used to establish the toxicity or lack of toxicity of the supplemented ration. The results of this series of experiments are given in table 2.

These data reveal that the toxicity of a liver and its vitamin A content are closely related. Furthermore, the amount of the vitamin ingested by rats which succumbed to the paralysis was in excess of the toxic dosage of 15,000 I.U. per day reported by Josephs ('44).

The results of the experiments with crystalline vitamin A alcohol and halibut liver oil supplements, summarized in table 2, have definitely identified the telang liver toxicity as a hypervitaminosis A.

The toxic effects of vitamin A supplements and of telang liver were identical. Bone fractures were observed in sixteen of the eighteen animals fed vitamin A supplements approximately equivalent to the intake of the vitamin by weanling rats on toxic telang liver rations. The two animals that failed to show symptoms of bone brittleness resisted the feeding of vitamin A supplements and did not always take the entire dose. All the constant symptoms observed in rats fed telang liver (growth failure, respiratory involvements, etc.) were reproduced in the animals receiving toxic doses of vitamin A.

DISCUSSION

It is clear from the results reported in this paper that the toxic effects resulting from the ingestion of very high amounts of telang liver are due to the accumulation of extra amounts of vitamin A in these livers. At present it is impossible to decide whether the telang liver lesions are the result of the abnormal storage of vitamin A or the increased storage is related to a disturbance in the structure of the liver due to other causes. The high vitamin A content makes the telang liver more valuable from a strictly nutritional point of view since a level high enough to produce hypervitaminosis A would never be used in ordinary rations.

The toxic dosage of vitamin A for growing rats which we have set at approximately 20,000 I.U. per day agrees favorably with the work reported by Rodahl and Moore ('43) and Josephs ('44).

SUMMARY

Telang and normal livers were quite similar in their content of carotene, ascorbic acid, choline, pantothenic acid, niacin, biotin and of

fat. The iron content of the telang livers was about 50% higher than observed in normal livers. The copper content of the two types of liver showed wider fluctuations but were of the same order.

About 20% of fresh telang livers tested were toxic to weanling rats receiving the calcium supplemented liver as their sole dietary. The symptoms observed in the affected rats were growth failure, paralysis and multiple spontaneous fractures of the bones of the extremities.

The toxicity of certain telang livers was due to their abnormally high stores of vitamin A. When these livers supplemented only with calcium carbonate were fed to rats, the daily intake of the vitamin was in excess of the toxic dosage of 15,000 I.U. per day. Equivalent amounts of crystalline vitamin A alcohol or of vitamin A supplied by halibut liver oil produced identical symptoms of toxicity when fed to weanling rats.

LITERATURE CITED

CARTA, A 1938 Indagini sulle macchie teleangectasiche del tegato dei bovini Profillassi, vol 11, pp 43–53.

DAVIES, W. A 1933 The colorimetric determination of vitamin A by the alkali digestion method Biochem J., vol 27, p 1770.

FREDERICK, L D 1943 The economic and nutritional importance of bovine hepatic disturbances. J Am Vet Med Assn, vol. 52, p 338.

HERBST, E J, P L PAVCEK AND C A ELVEHJEM 1944 Telang liver and vitamin A toxicity Science, vol 100, p 338

JOSEPHS, H. W. 1944 Hypervitaminosis A and carotenemia Am J Dis Child, vol 67, p. 33.

KANSAS STATE COLLEGE EXPERIMENT STATION 1940 Color in beef studies

MINDLIN, R. L., AND A. M BUTLER 1938 The determination of ascorbic acid in plasma A macromethod and micromethod. J. Biol. Chem, vol. 122. p. 673

RODAHL, K., AND T MOORE 1943 The vitamin A content and toxicity of bear and seal liver. Biochem. J., vol. 37, p. 166.

SCHOTE, F 1936 Beitrage zur Pathogenese der Telaeangiecktasie der Rinderleber Inaug. Diss Friedrich-Welhelma U of Berlin.

SMITH, H R 1940 Beef liver condemnations. Proc. 23rd ann meeting Am Soc. Animal Prod, p 272.

BIOMICROSCOPY OF THE EYES IN EVALUATION OF NUTRITIONAL STATUS. CONJUNCTIVAL CHANGES [1]

R. K. ANDERSON AND D. F. MILAM
North Carolina State Board of Health and Duke University School of Medicine, Durham

(Received for publication February 8, 1945)

During a survey of the nutrition status of a rural population of Wayne County, North Carolina, comprising 120 white families and 80 Negro families, with a total of 1,010 individuals (Milam and Anderson, '44), a study was made to determine whether there was any correlation between the severity and incidence of conjunctival changes detected in these persons by means of a slit lamp, and the level of vitamin A in their diet and in their blood plasma.

That vitamin A deficiency produces conjunctival changes characterized by xerosis, xerophthalmia, and keratomalacia has been recognized for many years. Kruse ('41) has described certain conjunctival changes occurring in 99% of a poor income group. These changes he considers as diagnostic of vitamin A deficiency. He observed clearing of these lesions in nine cases after prolonged therapy with very large doses of vitamin A (100,000 units daily for 6 months or more). Berliner ('42) has criticised Kruse's findings, stating that what Kruse calls Bitot spots are really pingueculae, and differ essentially from true Bitot spots in having the epithelium intact. Certainly Kruse's description more closely fits pingueculae than the usual description of Bitot spots. Kruse reported the highest incidence in older age groups, while true Bitot spots are said to be most common in children. Berliner ('43) could find no conjunctival changes in infants who were on a deficient diet and whose blood vitamin A values were well below normal; and he found such changes in persons with normal blood values. He believes that the changes described by Kruse are essentially degenerative changes associated with age and that pingueculae may be genetically determined.

[1] The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in cooperation with the North Carolina State Board of Health and Duke University School of Medicine.

Pett ('43) gave vitamin A for 3 months to thirty-two patients having conjunctival opacities and observed clearing in four of them. Sixty-three controls showed no change. Either the majority of the cases were not due to vitamin A deficiency or the period of therapy was not sufficiently prolonged.

Kruse divided his conjunctival lesions into three main types: those with gross spots, others with gross signs but no spots, and a third class with signs apparent only by biomicroscope. We have divided our cases into three approximately similar groups, referring to them as severe, moderate, and mild. However, it did not seem that the presence of a discrete spot was always the best criterion of severity; and occasional cases with small, grossly visible spots but with otherwise moderate or mild changes were put in the moderate groups and similarly, cases without discrete spots but with marked changes throughout a large portion of the conjunctiva were included in the severe group. When there was a question as to grouping, we tried to err on the side of conservatism, placing the case in a milder rather than a more severe group.

RESULTS AND DISCUSSION

Table 1 presents a comparison of blood vitamin A with conjunctival changes. In neither the white nor the colored group is there any indication that low blood vitamin A values are correlated with the presence of conjunctival changes. The lower values in the younger age group for those with mild changes are not significant from a statistical standpoint. Where values for all ages are combined there is a definite tendency for the blood values to be highest in those individuals with conjunctival abnormalities, a tendency which greatly decreases when the data are broken down into age groups. This is probably due largely to the fact that both blood vitamin A values and the severity of conjunctival changes tend to increase with age. It will be seen that the incidence of conjunctival changes shows a regular progression with increasing age to a point where a conjunctiva without changes in an adult is the exception.

Conjunctival changes appear to be more common in the Negroes than in whites, particularly in the younger age groups. In adults the incidence is so high in both races that the total incidence is not greatly different, but the severe grades are definitely more common in Negroes than in whites. This is in spite of the fact that in the families examined the average blood vitamin A values for the two races were not greatly different (whites, 95 I.U.; Negroes, 92 I.U.), and the dietary vitamin A, including carotene (white, 4,850 I.U.; Negroes, 5,540 I.U.), as well

TABLE 1

Comparison of plasma levels of vitamin A with severity of conjunctival changes.

AGE	CATEGORY OF INTEREST	WHITE RACE: Severity of conjunctival changes: None	Mild	Moderate	Severe	Per cent with conjunctival changes	Per cent with severe conjunctival changes	NEGRO RACE: Severity of conjunctival changes: None	Mild	Moderate	Severe	Per cent with conjunctival changes	Per cent with severe conjunctival changes
(years) 1–9	Number in group	60	6	0	0	9	0	58	14	2	0	22	0
	Mean plasma vitamin A	73	63					74	73	83			
	St. error of the mean	± 2.9	± 5 8					± 1.8	± 4 7	± 1.0			
10–15	Number in group	86	26	3	1	26	1	46	40	11	0	53	0
	Mean plasma vitamin A	81	78	76	94			86	87	87			
	St. error of the mean	± 2 5	± 4 2	± 2.4				± 3.3	± 2.7	± 3.8			
15 + Adult male	Number in group	5	19	45	32	95	32	4	16	15	55	96	62
	Mean plasma vitamin A	101	105	107	114			95	94	105	109		
	St. error of the mean	± 13.3	± 7.9	± 4.3	± 4 4			± 10.3	± 5.0	± 6 2	± 3.7		
15 + Adult female	Number in group	18	39	48	36	87	26	2	14	28	65	98	59
	Mean plasma vitamin A	90	88	100	98			82	97	84	92		
	St. error of the mean	± 4.0	± 3.6	± 3.7	± 4 2			± 8.8	± 6 7	± 4 2	± 3.1		
All ages	Number in group	169	90	96	69	60	16	110	84	56	120	70	32
	Mean plasma vitamin A	80	87	103	105			80	88	90	100		
	St. error of the mean	± 1.8	± 2.9	± 2.8	± 3.2			± 1.8	± 2.3	± 3.0	± 2.5		
Per cent persons with plasma vitamin A below 70 I.U.	Number in group	60	6	0	0			58	14	2	0		
	Age 4–9 yrs.	50%	67%		..			46%	57%	0%			
	Number in group	86	26	3	0			46	40	11	0		
	Age 10–15 yrs.	30%	38%					20%	18%	0%			
	Number in group	23	58	93	68			6	30	43	120		
	Adults 15 yrs +	17%	16%	11%	6%			0%	10%	21%	13%		
	Number in group	169	90	96	68			110	84	56	120		
	All ages	36%	26%	10%	6%			33%	21%	16%	13%		

TABLE 2

Comparison of mean vitamin A dietary intake with severity of conjunctival changes.

AGE	CATEGORY OF INTEREST	WHITE RACE				NEGRO RACE			
		Severity of conjunctival changes				Severity of conjunctival changes			
		None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
(years) –9	Number in group	56	6	0	0	56	10	2	0
	Mean vitamin A intake in international units	3550	3268			5709	3082	4915	
	St. error of the mean	± 232	± 842			± 690	± 666	± 1603	
0–15	Number in group	75	19	4	1	44	41	10	0
	Mean vitamin A intake in international units	4212	4856	9910	2334	4726	5945	8738	
	St. error of the mean	± 399	± 651	± 3220	.	473	680	1032	
15 + Adult male	Number in group	5	19	39	28	4	14	14	51
	Mean vitamin A intake in international units	2740	5074	6115	5340	6851	5651	6124	5835
	St. error of the mean	± 953	± 674	± 624	± 613	± 2190	± 1267	± 902	± 683
15 + Adult female	Number in group	18	36	47	31	3	13	28	56
	Mean vitamin A intake in international units	4726	4763	4560	5284	3887	6339	4177	5682
	St. error of the mean	± 669	± 525	± 368	± 770	± 882	± 971	± 477	± 753
All ages	Number in group	154	80	90	60	107	78	54	107
	Mean vitamin A intake in international units	3984	4747	5471	5261	5296	5591	5554	5755
	St. error of the mean	± 231	± 333	± 383	± 493	± 425	± 476	± 459	± 513
Per cent of persons with intakes below 2500 I.U. of vitamin A daily		27	28	17	25	24	23	22	29

as blood carotene (white, 286 I.U.; Negro, 422 I.U.) was definitely higher in the Negroes.

Table 2 presents a comparison of average daily vitamin A intakes with the conjunctival changes. Again there is no evidence of correlation with the incidence or severity of the conjunctival changes. Since the average vitamin A intake approaches the daily allowance recommended by the National Research Council, one would not expect an incidence of lesions of vitamin A deficiency as high as the incidence of conjunctival changes encountered in this study.

We realize that the exact interpretation of blood vitamin A values is still doubtful and that the blood values are by no means invariably correlated with the presence or severity of the clinical lesion. Probably even more than with dietary intakes, however, it would seem logical to expect some correlation between blood values and the signs of avitaminosis in a rather large group of persons. That correlation of both blood and dietary vitamin A with the conjunctival lesion is absent impairs seriously the validity of the view that a conjunctival lesion is evidence of vitamin A deficiency.

The inaccuracy, lack of sensitivity, and cumbersomeness of most of the present methods of evaluating nutritional status is fully recognized. We therefore feel some disappointment that slit lamp examination of the eyes has not thus far, in our experience, served as useful a purpose as was at first hoped.

SUMMARY

In a nutrition survey of a population group numbering 1,010, no correlation was observed between the severity and incidence of conjunctival changes detected with the slit lamp, and the level of vitamin A in the diet or in the blood plasma.

The frequency and severity of conjunctival changes were greater in the Negro than in the white.

LITERATURE CITED

BERLINER, M. L. 1942 Regarding early detection of avitaminosis A by gross or biomicroscopic examination of conjunctiva. Am. J. Ophth., vol. 25, p. 302.

——— 1943 Biomicroscopy of the eye. Harper, New York, vol. 1. pp. 159–168.

KRUSE, H. D. 1941 Ocular manifestations of avitaminosis A with special consideration of detection of early change by biomicroscopy. Pub. Health Rep., vol. 56, p. 1301.

MILAM, D. F., AND R. K. ANDERSON 1944 Nutrition survey of an entire rural county in North Carolina. South. Med. J., vol. 37, p. 597.

PETT, L. B. 1943 Riboflavin and vitamin A in relation to eye strain. Canad. Med Assn. J., vol. 49, p. 293.

BIOMICROSCOPY OF THE EYES IN EVALUATION OF NUTRITIONAL STATUS. CORNEAL VASCULARIZATION [1]

R. K. ANDERSON AND D. F. MILAM
North Carolina State Board of Health and Duke University School of Medicine, Durham

(Received for publication February 8, 1945)

There is good experimental and clinical evidence that certain vitamin deficiencies produce pathological changes in the superficial structures of the eye. Vascularization of the cornea in riboflavin deficiency in rats was demonstrated by Bessey and Wolbach ('39) and in humans by Sydenstricker et al. ('40).

Although few students of the problem seriously doubt the production of corneal invasion by riboflavin deficiency, the specificity of this sign has been seriously questioned by recent work. Sandstead ('42) was unable to produce regression of corneal vascularization with large doses of riboflavin (15 mg. daily for 60 days). Scarborough ('42) observed no improvement under riboflavin therapy except in persons with other evidence of deficiency disease. Youmans et al. ('42) found no correlation of corneal vascularization with dietary intake of riboflavin or with other probable signs of riboflavin deficiency. Machella and McDonald ('43) saw some temporary improvement under riboflavin therapy, but all their patients relapsed even while riboflavin was being taken. Pett ('43) observed a high percentage of clearing under riboflavin therapy (57%) but noted also a large percentage of clearing in the control group (42%). Tisdall et al. ('43) reported definite improvement in the majority of patients with severe degrees of invasion. Their study was made with aviators subjected to rather unusual light conditions; the severe cases which they treated were possibly more likely to have been caused by riboflavin deficiency than the milder degrees more commonly seen.

Subsistence of humans on diets presumably deficient with regard to riboflavin has not been observed to result in corneal vascularization.

[1] The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in cooperation with the North Carolina State Board of Health and Duke University School of Medicine.

Sebrell and Butler ('38, '39) in their production of clinical riboflavin deficiency in man did not observe corneal changes. Boehrer et al. ('43) were unable to find corneal vascularization in six persons who received only 0.47 mg. of riboflavin daily in their diet for a period of 5 weeks, although one of the control subjects developed this condition, which persisted for 5 weeks in spite of a normal diet and 9 mg. of riboflavin daily. Williams et al. ('43) failed to observe vascularization or other evidence of riboflavin deficiency in four patients who had subsisted on a diet containing 0.21 mg. riboflavin per 1,000 cal. for 288 days.

Vail and Ascher ('43) emphasize the presence of a normal pericorneal plexus of vascular arcades and believe that much of the so-called corneal vascularization is only engorgement of this normal pericorneal plexus. They prefer the term "concentric collateral vessels." They observed a higher incidence of this condition in patients taking a good diet than in those taking a poor one. They observed true corneal invasion only in patients with evidence of previous corneal disease. They feel that to regard "concentric collaterals" as a specific sign of riboflavin deficiency does not seem justified, although it might be a factor in some cases.

Corneal vascularization has been observed in several other deficiency states, namely vitamin A deficiency (Bessey and Wolback, '39), tryptophan deficiency (Trotter and Day, '42; Albanese and Buschke, '42; and Albanese et al., '43), lysine deficiency (Trotter and Day, '42), zinc deficiency (Follis et al., '41), sodium deficiency (Follis et al., '42), and deficiency of an unidentified component of the vitamin B complex (Gyorgy et al., '42). Corneal vascularization has also been reported to be produced by administration of thallium (Ginsberg and Buschke, '23) and of large doses of nicotinic acid (Gregory, '43). The question of the validity of corneal invasion as a measure of riboflavin deficiency has been the subject of several recent reviews (Nutrition Reviews, '43, '44).

Because of the accessibility of the superficial eye structures, their examination by means of the slit lamp or biomicroscope has been recommended, particularly by Kruse ('42), as a convenient and accurate method of detecting mild degrees of deficiency of riboflavin and vitamin A.

In connection with a survey of the nutritional status of the population of the rural section of Wayne County, North Carolina, which was carried out between July 1942 and June 1943, and included 120 white families and 80 Negro families (a total of 1,010 individuals), we made slit lamp examinations of the eyes of all persons who were capable of

cooperating in such a test. This paper discusses the incidence of corneal vascularization in these persons, as compared with their riboflavin intake. A report of the general aspects of the survey has been published elsewhere (Milam and Anderson, '44). The families included in the survey were selected on the basis of statistical principles to represent correct proportions from geographical areas and from racial and economic groups.

METHODS

The persons given the slit lamp test were, as a rule, over 4 years old, since satisfactory examinations of this kind were usually not possible in children under that age. From a 7-day food intake record on each person, the daily average intake of the various nutrients was calculated. Corneal invasion was classified as follows:

1. Increased vascularity, and engorgement of the limbic plexus, with some capillaries just appearing to cross the sclero-corneal junction. We have found it difficult to be certain always of the exact location of this junction, capillaries at times appearing to be in the cornea when the slit light was directed at one angle and in the very thin portion of sclera at the margin of the cornea when the light was at a different angle. Vail and Ascher ('43) have emphasized the fact that the final vessels may appear to be in the corneal tissues when they are really only in the transparent tissue that forms the conjunctivo-scleral wedge. There have been included in this group 1, however, only those cases where the capillaries appeared quite definitely to cross the scleral margin.
2. Invasion of the cornea by capillaries to a considerable distance, but with penetration less than a fourth of the distance from the edge of the cornea to its center.
3. Invasion of the cornea by capillaries to a distance between a fourth and a half that to the center of the cornea.
4. Invasion of the cornea by vessels half or more of the distance to its center. Only one case with this degree of severity was seen in the survey.

The degree of penetration of the blood vessels is not the only measure of the severity of corneal vascularization. Certainly the extent of the periphery of the cornea involved and the amount of proliferation and engorgement of vessels is also of importance. For purposes of classification, however, the degree of penetration seems to be the most reliable and useful criterion.

RESULTS AND DISCUSSION

Data concerning the incidence of corneal invasion for various age groups of the white and colored persons, as compared with their mean riboflavin intake were obtained (table 1). It will be noted that there is no correlation between the riboflavin intake and the incidence or severity of the corneal invasion. The one white adult female with grade 3 invasion did have a low riboflavin intake; but no great significance can be attached to this one case, particularly when persons with even lower intakes were found in all the other groups. The incidence of invasion in the white persons is high in all age groups, the least incidence being in adult males and the highest in the 10- to 15-year age group, although the significance of these differences may be questioned.

The most striking feature apparent in a comparison of the two classes is the markedly lower incidence of corneal invasion in the Negro. As among the whites, the highest incidence in Negroes is in the 10- to 15-year age group and the lowest is in adult males. We have no adequate explanation for this difference in incidence between Negroes and whites. Youmans et al. ('42) also observed the markedly lower incidence in the Negro and suggest that this relative freedom from corneal invasion may be related to their pigmentation. One factor in the adult group may be the higher incidence of arcus senilis in the Negro. In our survey this lesion appeared about eight times as frequently in the Negro as in the white. Sydenstricker et al. ('40) have commented on the absence of corneal invasion in persons with an arcus, and we have had the same experience. However, the incidence of an arcus in our study was not sufficient to have any great effect on the incidence of vascularization. One wonders whether the marked difference observed by Vail and Ascher ('43) in the incidence of vascularization in Cincinnati (45%), as compared with that in Birmingham (13%), may not have been partially due to the greater Negro population in the latter region.

There is a greater difference in the mean riboflavin intakes for the various groups of Negroes than for the groups of whites, due partially at least to the small number of cases in some of the positive groups. However, none of these differences could be shown to be statistically significant.

Although the number is too small to permit conclusions, it may be significant that the three Negroes with grades 3–4 invasion had low intakes of riboflavin. We feel that in the absence of definitive eye disease, the severer grades of invasion are much more likely to be caused by

TABLE 1

Comparison of riboflavin dietary intake (in µg.) with incidence of varying degrees of corneal vascularization.

AGE	CATEGORY OF INTEREST	WHITE RACE						NEGRO RACE						
		Extent of corneal invasion				Per cent with invasion	Per cent with grade II and III invasion	Extent of corneal invasion					Per cent with invasion	Per cent with grade II and III invasion
		None	I	II	III			None	I	II	III	IV		
(years)														
4–9	Number in group	24	29	9	0	61	15	55	10	2	0	0	18	3
	Mean riboflavin intake in micrograms	924	983	991				825	921	689				
	St. error of the mean	± 75	± 24	± 122				± 52	± 101	± 230				
10–15	Number in group	24	47	21	9	76	36	67	22	3	1	0	28	4
	Mean riboflavin intake in micrograms	1144	1160	1192	984			1052	1003	1242	508			
	St. error of the mean	± 115	± 88	± 120	± 147			± 58	± 149	± 328				
15 + Adult male	Number in group	38	43	12	0	59	13	77	3	2	1		7	4
	Mean riboflavin intake in micrograms	1280	1314	1050				984	1731	1192	624			
	St. error of the mean	± 89	± 116	± 159				± 58	± 297	± 130	..			
15 + Adult female	Number in group	50	55	23	1	61	19	82	14	5	0	1	20	6
	Mean riboflavin intake in micrograms	945	1047	937	703			845	1090	830		665		
	St. error of the mean	± 60	± 69	± 94				± 49	± 150	± 124				
All ages	Number in group	136	174	65	10	65	20	281	49	12	2	1	19	4
	Mean riboflavin intake in micrograms	1070	1133	1048	956			929	1056	970	566	665		
	St. error of the mean	± 43	± 44	± 62	± 138			± 28	± 88	± 126				
Per cent of persons with riboflavin intakes below 1 mg daily		52	50	55	60			62	61	58	100	100		

riboflavin deficiency than the milder types so commonly seen. However, many persons with still lower intakes had no invasion.

The one person with grade 4 invasion also had an angular cheilosis suggestive of riboflavin deficiency. Twelve other persons seen in this survey had angular cheilosis suggestive of riboflavin deficiency. Of these, 50% had no vascularization of the cornea and the rest had grade 1 only. We do not consider that cheilosis is a pathognomonic sign of riboflavin deficiency, but we believe that it is more likely to indicate such deficiency than is corneal invasion. Unfortunately, conditions in this survey were not favorable to a therapeutic test, but in other work we have seen persons without corneal invasion who had rather severe angular cheilosis which responded to riboflavin therapy. We have also seen persons with both cheilosis and corneal invasion in which the cheilosis responded to riboflavin therapy and the invasion did not.

There were nineteen white and nine colored persons who showed tongue changes suggestive of vitamin B complex deficiency. Of the nineteen whites, seven had grade 1, four had grade 2, and one had grade 3 corneal invasion; the rest had none. Eight of the nine colored persons were examined by slit lamp. Six showed no invasion, one showed grade 1 invasion, and the other had grade 2 invasion.

We do not doubt that riboflavin deficiency can produce corneal invasion and that in some instances, along with other evidence of riboflavin deficiency, this sign may furnish confirmatory evidence of the deficiency. However, it appears doubtful whether any very large percentage of cases, particularly those of milder degree so commonly seen, are due to the lack of riboflavin. We realize that there are many unavoidable errors encountered in the taking and calculating of diet records and that even if the diet record is entirely accurate, the recent dietary intake does not always reflect the previous intake responsible for the avitaminosis. Nevertheless, in a large group of individuals, some correlation between the presence of an avitaminotic lesion and the dietary intake is to be expected. Its complete absence in the persons included in our study casts doubt on the value of corneal invasion as a measure of riboflavin deficiency in population groups. The additional fact that there was no correlation between corneal invasion and other physical signs usually considered as suggestive of riboflavin deficiency, also leads us to doubt the diagnostic value of corneal invasion as the sole criterion of riboflavin deficiency. The development of corneal vascularization on diets low but not exceedingly low in riboflavin, as in this study, may well depend on the presence or absence of other

conditioning factors which were not recorded in this study. The search for such factors would be an enterprise well worth while.

SUMMARY

In a nutrition survey of a rather large population group, no correlation could be found between corneal invasion and the level of riboflavin in the diet. There was also no correlation with other signs suggestive of riboflavin deficiency.

Corneal invasion was found to be much more frequent in the white race than in the Negro race.

LITERATURE CITED

ALBANESE, A. A., R. M. RANDALL AND L. E. HOLT, JR. 1943 Effect of tryptophane deficiency on reproduction. Science, vol. 97, p. 312.

ALBANESE, A. A., AND W. BUSCHKE 1942 On cataract and certain other manifestations of tryptophane deficiency in rats. Science, vol. 95, p. 584.

BESSEY, O. A., AND S. B. WOLBACH 1939 Vascularization of the cornea of the rat in riboflavin deficiency, with note on corneal vascularization in vitamin A deficiency. J. Exp. Med., vol. 69, p. 1.

BOEHRER, J. J., C. E. STANFORD AND E. RYAN 1943 Experimental riboflavin deficiency in man. Am. J. Med. Sci., vol. 205, p. 544.

FOLLIS, R. H., JR., E. ORENT-KEILES AND E. V. MCCOLLUM 1942 Histologic studies of tissue of rats fed a diet extremely low in sodium. Arch. Path., vol. 33, p. 504.

FOLLIS, R. H., JR., H. G. DAY AND E. V. MCCOLLUM 1941 Histological studies of tissues of rats fed a diet extremely low in zinc. J. Nutrition, vol. 22, p. 223.

GINSBERG, S., AND A. BUSCHKE 1923 Ueber die Augenveränderungen bei Ratten nach Thalliumfütterung (Katarakt und Iritis) und ihre Beziehungen zum endokrinen System. Klin. monatsbl. f. Augenh. Stuttg., vol. 71, p. 385.

GREGORY, M. K. 1943 Ocular criteria of deficiency of riboflavin. Brit. Med. J., vol. 2, p. 134.

GYORGY, P., R. E. ECKARDT AND L. V. JOHNSON 1942 Biological action of the vitamins. (E. A. Evans, ed.), Univ. Chicago Press, p. 64.

KRUSE, H. D. 1942 A concept of the deficiency states. Milbank Mem. Fund Quart., vol. 20, p. 245.

MACHELLA, T. E., AND P. R. MCDONALD 1943 Studies of B vitamin in human subject; failure of riboflavin therapy in patients with accepted picture of riboflavin deficiency. Am. J. Med. Sci., vol. 205, p. 214.

MILAM, D. F., AND R. K. ANDERSON 1944 Nutrition survey of an entire rural county in North Carolina. South. Med. J., vol. 37, p. 597.

NUTRITION REVIEWS 1943 Vol. 1, pp. 194, 327.

———— 1944 Vol. 2, pp. 120, 139.

PETT, L. B. 1943 Riboflavin and vitamin A in relation to eye strain. Canad. Med. Assn. J., vol. 49, p. 293.

SANDSTEAD, H. R. 1942 Superficial vascularization of cornea; result of riboflavin therapy. Pub. Health Rep., vol. 57, p. 1821.

SCARBOROUGH, H. 1942 Circumcorneal injection as a sign of riboflavin deficiency in man, with an account of three cases of ariboflavinosis. Brit. Med. J., vol. 2, p. 601.

SEBRELL, W. H., AND R. E. BUTLER 1938 Riboflavin deficiency in man; preliminary note. Pub. Health Rep., vol. 53, p. 2282.

——— 1939 Riboflavin deficiency in man (ariboflavinosis). Pub. Health Rep., vol. 54, p. 2121.

SYDENSTRICKER, V. P., W. H. SEBRELL, H. M. CLECKLEY AND H. D. KRUSE 1940 Ocular manifestations of ariboflavinosis; progress note. J. Am. Med. Assn., vol. 114, p. 2437.

TISDALL, F. F., J F. MCCREARY AND H. PEARCE 1943 Effect of riboflavin on corneal vascularization and symptoms of eye fatigue in R. C. A. F. personnel. Canad. Med. Assn. J., vol. 49, p 5.

TROTTER, J. R., AND P. L. DAY 1942 Cataract and other ocular changes resulting from tryptophane deficiency. J. Nutrition, vol. 24, p. 159.

VAIL, D, AND K. W. ASCHER 1945 Corneal vascularization problems. Am. J. Ophth., vol. 26, p. 1025.

WILLIAMS, R. D, H. L. MASON, P. L. CUSICK AND R. M. WILDER 1943 Observations on induced riboflavin deficiency and riboflavin requirement of man. J. Nutrition, vol. 25, p. 361.

YOUMANS, J. B., E. W. PATTON, W. D. ROBINSON AND R. KERN 1942 Analysis of corneal vascularization as found in survey of nutrition. Tr. Assn. Am. Physicians, vol. 57, p. 49.

THIAMINE, RIBOFLAVIN AND NICOTINIC ACID RETENTION IN PREPARATION OF OVERSEAS HAMS AND BACONS[1]

HERBERT P. SARETT AND VERNON H. CHELDELIN
Department of Chemistry, Oregon State College, Corvallis

(Received for publication March 23, 1945)

The present report is based on a study of retention of thiamine, riboflavin and nicotinic acid in the preparation of overseas smoked hams and bacons. These hams and bacons were more heavily salted than the ordinary commercial products to prevent deterioration in shipping to the armed forces. The study was undertaken to study vitamin retention after soaking and cooking of these cured meats according to the directions issued by the Office of the Quartermaster General. A study of vitamin losses in cooking of regular commercially cured hams has been reported by Schweigert, McIntire and Elvehjem ('43).

EXPERIMENTAL

Five overseas hams, weighing 13–14 lbs. each, were prepared according to the army directions and samples taken for analysis at each step. Care was taken to obtain each time at least two large representative samples from each ham. Three of the hams were prepared as whole hams, two as sliced hams. The directions for soaking and cooking were as follows:

Whole ham. Scrub, scrape or brush to remove mold. Trim as necessary (weight). Cover ham with warm water and let soak over night. Drain. Rinse salty sediment from kettle. Cover ham with water. Bring to boiling point, then simmer for at least 3 hours, changing the water at end of each hour. Rinse salty sediment from bottom of kettle each time. During final hour of cooking, add 1 pound of sugar to water in which ham is simmering. Remove ham from water. Skin. Serve boiled, baked, sliced and fried. Baked ham: Place ham in pan, fat side up.

[1] This study was conducted with the cooperation of and published with the approval of the Office of the Quartermaster General, and was supported by the Committee on Food Composition of the National Research Council and the Nutrition Foundation, Inc.

Published with the approval of the Monographs Publication Committee, Oregon State College. Research paper no. 92, School of Science, Department of Chemistry.

Roast uncovered at 300°F. (slow oven) allowing 18 to 25 minutes to the pound. Fried ham: Place slices of ham in hot frying pan and cook in their own fat until brown on both sides.

Sliced ham. Scrub, scrape or brush to remove mold Slice into ½-inch slices. Trim, removing rind and excess fat. Soak slices of ham in warm water for 4 hours, changing the water every hour and rinsing salty sediment from bottom of kettle each time. Remove ham slices from water. Drain well. Bake or fry slices of ham.

The hams were weighed after each process to correct for changes in weight. Vitamin retentions are based upon original and final weights of the ham, with appropriate correction for the weight of the bones. The drippings from baked ham were retained for analysis. The whole hams were baked 18, 21, and 25 minutes per pound, respectively, to show possible differences due to cooking time. These are reflected in the extreme range of vitamin content of the drippings.

Bacon. Six bacon slabs, weighing 14–16 lbs. each, were prepared according to the following directions: Cut bacon into slices (7 or 8 per inch) and soak in solution of sugar for 2 hours before frying. Use 1 gallon of water containing 1.5 lbs. of sugar for each 18 lbs. of bacon. When frying, care should be taken not to overcook.

Samples of bacon were analyzed as the original cuts, after soaking, and after soaking and frying. Representative samples (7–12 slices) were obtained by combining each third slice of serial cuts for each of the above analyses. Two sections of two bacons were analyzed separately, and two sets of pooled slices from the remaining four bacons taken for assay. The bacon samples were weighed after each process, as was done with the hams. The changes in weight were taken into account in calculating vitamin retention.

METHODS

All samples, except drippings, were ground four times in a meat grinder prior to analysis. Thiamine assays were made on enzymatically digested samples, using Lactobacillus fermenti (Sarett and Cheldelin, '44). Samples for riboflavin analysis were autoclaved with 0.1N HCl for 15 minutes (Strong and Carpenter, '42) and assayed by the method of Snell and Strong ('39), using the glucose and acetate concentrations suggested by Stokes and Martin ('43). Samples were digested for nicotinic acid analysis by autoclaving for 30 minutes with 1N H_2SO_4 and, after neutralization and filtration, assayed using Lactobacillus arabinosus 17–5 by the method of Sarett, Pederson and Cheldelin ('45). The extraction procedures and the assay methods used have been thoroughly

checked and have been found to give valid results as compared to other available techniques.

RESULTS

In table 1 the results on the hams and bacons are summarized. The vitamin content at each stage of preparation is given and the per cent retention calculated on the basis of the original and final weights of the sample. Although only two sliced hams were used in the study, ranges and average figures are given for these, since two samples were taken from each ham.

The overnight soaking of whole hams removed very little thiamine, riboflavin or nicotinic acid. However, the subsequent boiling reduced the average retention of these three vitamins to 72, 79, and 79% respectively. Slices of boiled ham which were then fried, lost another 10–15% of the original content of each of the three vitamins, whereas baking of the whole ham destroyed about 20% of the thiamine but had very little effect on the other vitamins. The drippings contained only a few per cent of any of the vitamins. The wide variation in vitamin content of the drippings may have been due in part to the difference in baking time for each of the three hams, i.e., 18, 21, and 25 minutes per pound. The baked ham, exclusive of drippings, retained 50, 79, and 71% of the original thiamine, riboflavin and nicotinic acid, respectively.

The greatest loss of vitamins with sliced ham was in the preliminary soaking, where reductions in thiamine, riboflavin and nicotinic acid content averaged 41, 15, and 42%, respectively. Baking of these soaked slices caused a little more destruction of riboflavin than frying, while the losses of thiamine and nicotinic acid were about the same for both cooking methods. The thiamine, riboflavin and nicotinic acid retention in baked slices of ham were 47, 68, and 47%, respectively.

Schweigert, McIntire and Elvehjem ('43) studied vitamin losses in cooking commercially cured hams. They found about the same concentration of nicotinic acid in cured hams as is reported here, but less thiamine and riboflavin. They report retentions after roasting of 58, 74 and 79% for thiamine, riboflavin, and nicotinic acid, respectively. These figures agree with the vitamin content reported here for baked ham, 50, 79, and 71%. However, Schweigert et al. ('43) also found 10–15% of the vitamins in the drippings in contrast to the small amounts observed in the present paper. The high vitamin content of those drippings appears to correspond to the losses we observed in the preliminary soaking and boiling of the overseas hams and may represent a portion of the vitamin which is less firmly bound than that which is retained in the cooked meat.

TABLE 1

Thiamine, riboflavin and nicotinic acid retention in soaking and cooking of overseas hams and bacons.

SAMPLE	WEIGHT RETENTION, PER CENT		VITAMIN CONTENT IN µG. PER GM.						VITAMIN RETENTION IN PER CENT					
			Thiamine		Riboflavin		Nicotinic acid		Thiamine		Riboflavin		Nicotinic acid	
	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.
Whole ham (3)														
As received			8.5–10.6	10.1	2.0–2.9	2.5	20.0–40.8	31.1						
Soaked	100–102	101	8.1–11.6	9.5	1.9–2.6	2.2	18.6–40.0	30.5	81–100	94	90–95	93	94–99	97
Boiled	82–92	86	7.3– 9.7	8.5	1.9–2.2	2.1	16.7–34.6	28.8	59–83	72	66–95	79	69–93	79
Slices, fried	55–71	64	7.6– 9.0	8.3	2.3–2.7	2.5	27.3–35.4	32.5	49–58	55	55–75	67	61–75	68
Baked	53–63	61	7.1–10.1	8.2	2.7–3.2	3.0	28 –44.2	35.3	43–58	50	69–85	79	66–74	71
Drippings, baked	6–11	8	0 – 5.0	3.0	0.6–1.1	0.8	4.8–48.4	25.0	0–4	2	2–4	3	2–8	6
Sliced ham (2)														
As received			9.5–11.6	10.8	2.3–2.5	2.4	24.3–27.2	25.5						
Soaked	100–105	102	6.0– 6.3	6.2	1.9–2.1	2.0	13.6–14.9	14.4	54–69	59	76–91	85	55–61	58
Fried	55–75	65	7.0– 8.6	7.7	2.7–3.3	2.9	18.5–22.1	20.4	40–57	47	75–80	78	45–61	52
Baked	59–70	65	7.1– 9.0	7.8	2.3–2.9	2.5	16.2–19.7	18.4	42–56	47	64–71	68	46–50	47
Drippings, baked	19, 10		1.3, 6.7		0, 1.6		12.7, 11.7		2, 7		0, 7		10, 5	
Bacons (6)														
As received			2.7– 4.1	3.3	0.9–1.2	1.0	11.5–14.5	12.6						
Soaked	104–106	105	1.3– 2.2	1.7	0.6–1.1	0.75	4.2– 7.9	5.5	47–77	52	69–100	78	36–69	42
Fried	22–31	25	1.7– 5.5	2.7	2.1–3.9	2.85	10.4–19.6	14.7	15–41	20	61–100	72	21–40	30
Drippings, fried	56–57	56	0, 0		0, 0		1.7, 0.9		0, 0		0, 0		8, 4	

After frying sliced ham, Schweigert et al. ('43) found a retention of 86, 77, and 85%, respectively, of thiamine, riboflavin, and nicotinic acid, and 7–10% more in the drippings. Since the slices we used for frying had already lost part of their vitamin content in previous soaking, or soaking and boiling, it is difficult to compare the present results with their figures. However, it can be seen from the data of table 1 that although the final vitamin retentions for fried ham were low, the actual percentages lost in the frying alone are only from 12–17% for thiamine, 0–8% for riboflavin, and 6–11% for nicotinic acid.

The soaking of sliced bacon removed 48, 22, and 58% of the original thiamine, riboflavin, and nicotinic acid content, respectively. Frying had little further effect on the riboflavin content but destroyed a little more nicotinic acid and most of the thiamine. Only 20% of the original thiamine remained which is comparable to the 13% retention reported for one sample of ordinary bacon by Lane, Johnson, and Williams ('42). However, the 72 and 30% remaining of the riboflavin and nicotinic acid respectively are quite lower than the values of about 100% retention for these vitamins reported by Cheldelin, Woods, and Williams ('43) on unsoaked samples. The major portion of the loss of these two vitamins shown here was in the preliminary soaking. A small part (only a few per cent) of the nicotinic acid was present in the drippings, but no thiamine or riboflavin was found.

SUMMARY

The thiamine, riboflavin and nicotinic acid contents of heavily salted overseas hams and bacons were studied at different stages of cooking.

Average retentions of thiamine, riboflavin, and nicotinic acid after soaking and boiling of whole hams were 72, 79, and 79%, respectively. Subsequent frying reduced the remaining vitamins to 55, 67, and 68%, respectively, whereas after baking the comparable figures were 50, 79, and 71%. Only a small part (few per cent) of any of the vitamins were found in drippings of the baked ham.

Soaking of sliced ham left only 59, 85, and 58%, respectively, of the original thiamine, riboflavin, and nicotinic acid contents. Retentions after frying these slices were 47, 78, and 52%, while after baking 47, 68, and 47%, respectively, of the thiamine, riboflavin, and nicotinic acid were found.

The losses of these vitamins on soaking of overseas bacon were large, leaving only 52, 78, and 42% of the original thiamine, riboflavin, and nicotinic acid. The amounts of these vitamins remaining after frying were 20, 72, and 30%, respectively.

ACKNOWLEDGMENT

The authors wish to thank Margaret J. Bennett and Thomas R. Riggs for their technical assistance.

LITERATURE CITED

CHELDELIN, V. H., A. M. WOODS AND R. J. WILLIAMS 1943 Losses of B vitamins due to cooking of foods. J. Nutrition, vol. 26, p. 477.

LANE, R. L., E. JOHNSON AND R. R. WILLIAMS 1942 Studies of the average American diet. I. Thiamine content. J. Nutrition, vol. 23, p. 613.

SARETT, H. P., AND V. H. CHELDELIN 1944 The use of Lactobacillus fermentum 36 for thiamine assay. J. Biol. Chem., vol. 155, p. 153.

SARETT, H. P., R. L. PEDERSON AND V. H. CHELDELIN 1945 Microbiological assay methods for nicotinic acid. Arch. Biochem. (in press).

SCHWEIGERT, B. S., J. M. MCINTIRE AND C. A. ELVEHJEM 1943 The retention of vitamins in meats during storage, curing and cooking. J. Nutrition, vol. 26, p. 73.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. and Eng. Chem., Anal. Ed., vol. 11, p. 346.

STOKES, J. L., AND B. B. MARTIN 1943 Influence of buffer and glucose in the Lactobacillus casei assay for pantothenic acid. J. Biol. Chem., vol. 147, p. 483.

STRONG, F. M., AND L. E. CARPENTER 1942 Preparation of samples for microbiological determination of riboflavin. Ind. and Eng. Chem., Anal. Ed., vol. 14, p. 909.

THE RIBOFLAVIN, NIACIN AND THIAMINE CONTENT OF DRIED LEGUMINOUS SEEDS[1]

LOUISE DANIEL AND L. C. NORRIS
School of Nutrition, Cornell University, Ithaca, New York

(Received for publication February 12, 1945)

Recent world conditions have given a considerable impetus to the use of legumes as a source of dietary protein. Because of this their vitamin content has become of interest. A review of the literature disclosed certain definite gaps in our knowledge of the B-vitamin content of legumes. With the exception of soybeans, blackeye peas and peanuts very few data are to be found; especially is this true of riboflavin values, and to a lesser extent of niacin values. Moreover, some of the results recorded may be considered questionable, since the analyses were performed before the introduction of the more accurate chemical and microbiological procedures. In view of this a study has been made to determine the riboflavin, niacin and thiamine content of dried leguminous seeds using recent and improved methods.

Some of the more important papers on the vitamin content of legumes appearing in the literature are presented by Fixsen and Roscoe ('38), Booher and Hartzler ('39), Munsell ('40, '42, '43), Halverson and Sherwood ('40), Kelly, Dietrich and Porter ('40), Higgins, Holley, Pickett and Wheeler ('41), Sherman ('41), Booher, Hartzler and Hewston ('42), Teply, Strong and Elvehjem ('42), Burkholder ('43), Russell, Taylor and Beuk ('43).

EXPERIMENTAL

Samples of dried, unprocessed legumes were obtained from Agronomy Departments of many universities throughout the United States, from seed dealers, and on the open market. All samples were ground in a Wiley Mill using a 1-mm. sieve. Each sample was analyzed for riboflavin, niacin and thiamine. Two different methods were used for determining each vitamin in one sample of every variety of legume. Greater confidence is placed in the value obtained for a vitamin assay, if the results from two methods for determining the vitamin do not vary by more than 10%.

[1] The authors are indebted to Swift and Company for a grant in support of this work.

The riboflavin methods used were the fluorometric method of Hodson and Norris ('39), as modified by Scott ('43) and the microbiological procedure of Snell and Strong ('39). The extraction of the sample for the microbiological procedure was done by digestion with takadiastase and papain according to Cheldelin, Eppright, Snell and Guirard ('42). To free the aqueous extracts of fatty material, a petroleum ether extraction was carried out at a pH of 6.7, followed by autoclaving and filtration before placing the aliquots in the assay tubes. Inconsistent and high results were obtained unless the extracts were absolutely clear.

In the determination of niacin the microbiological method of Snell and Wright ('41) and the chemical procedure of Dann and Handler ('41) were used. The extracts for the microbiological method were prepared by the same procedure as that used in the riboflavin assay. The medium was modified to include 2 μg. per tube of p-aminobenzoic acid, as suggested by Isbell ('42) when using charcoal-treated casein hydrolyzate. Low blanks were obtained by washing the Labco casein with alcohol, refluxing for 20 hours with conc. HCl and treating with Darco G-60 for 2 hours. In the chemical procedure the extracts were completely colorless before the addition of cyanogen bromide.

The methods used for the determination of thiamine were the thiochrome and the yeast fermentation procedures. The thiochrome method was that proposed by the Research Corporation Committee on the Thiochrome Method, D. J. Hennessy, Chairman ('42). The fermentation method of Schultz, Atkin and Frey ('42) was used with the following modification.

The unknown solution was run at two different levels, along with two other tubes containing the same levels of unknown which had been treated with sodium sulfite to destroy thiamine. Two micrograms of thiamine were added to each of the sulfite blanks, in order to bring the amount of gas produced within the same range as that of the unknown. Two standard tubes containing 2 and 4 μg. of thiamine, respectively, were used. The difference between the sulfite blanks was subtracted from the difference between the two levels of unknown, thus giving the gas produced by thiamine alone in a given amount of sample. These results agreed well with the values obtained by the thiochrome method.

RESULTS AND DISCUSSION

A comparison of the results obtained by using two methods of analysis for each of the vitamins is presented in table 1. There is excellent agreement in all cases. The average percentage differences between the

methods for riboflavin, niacin and thiamine, respectively, are 2.67, 2.97 and 4.48. No definite positive or negative deviation in any of these sets of data was observed.

In view of these comparative data, it was thought justifiable to use only one method for the routine analysis of each vitamin. Scott's modification of the Hodson and Norris fluorometric method for determining riboflavin was chosen because it requires less time than the microbiological method, and because it eliminates the need for the removal of sub-

TABLE 1

Comparison of results obtained by two methods of analysis for riboflavin, niacin and thiamine.[1]

SAMPLE	RIBOFLAVIN			NIACIN			THIAMINE		
	Fluorometric	Microbiological	Difference	Microbiological	Chemical	Difference	Thiochrome	Yeast fermentation	Difference
	µg./gm.	µg./gm.	%	µg./gm.	µg./gm.	%	µg./gm.	µg./gm.	%
Red kidney beans	2.16	2.08	3.70	25.8	25.8	0.00	5.90	6.24	5.45
White marrow beans	1.58	1.62	2.47	25.9	26.2	1.15	5.42	5.85	7.35
Pea beans									
Robust	1.89	1.86	1.59	29.9	30.4	1.65	4.78	4.37	8.58
Michelite	1.74	1.67	4.02	27.1	27.1	0.00	6.34	6.35	0.16
Great Northern beans	1.90	1.88	1.05	18.2	19.2	5.21	7.85	8.78	10.59
Yelloweye beans	1.78	1.72	3.37	24.8	23.8	4.03	9.00	9.25	2.70
Pinto beans	2.16	2.20	1.82	17.7	18.0	1.67	7.16	6.80	5.03
Lima beans									
Baby	1.75	1.76	0.57	26.3	27.8	5.40	6.52	6.80	4.12
Fordhook	1.70	1.65	2.94	23.0	22.4	2.61	3.66	3.92	6.63
Large flat	1.70	1.72	1.16	22.1	23.7	6.75	4.00	4.34	7.83
Blackeye peas	2.08	2.12	1.89	28.8	28.1	2.43	5.56	5.85	4.96
Soybeans									
Chief	3.02	2.96	1.99	25.8	26.4	2.27	10.62	10.45	1.60
Richland	3.58	3.39	5.31	34.8	35.7	2.52	12.53	13.66	8.27
Manchu	3.92	3.95	0.76	37.0	36.4	1.62	9.95	10.00	0.50
Illini	3.15	3.09	1.90	23.4	24.4	4.10	11.10	11.00	0.90
Bansei	3.50	3.68	4.89	29.8	30.3	1.65	7.64	7.85	2.68
Split peas									
Yellow	1.59	1.70	6.47	28.4	28.9	1.73	6.86	7.47	8.17
Green	1.74	1.75	0.57	36.2	37.1	2.43	8.01	8.15	1.72
White kidney beans	1.93	1.85	4.15	31.2	30.2	3.21	6.34	6.32	0.32
Cranberry beans	2.58	2.60	0.77	24.4	25.2	3.17	5.61	5.47	2.50
Pink beans	2.43	2.36	2.88	19.8	19.8	0.00	8.10	7.78	3.95
Black turtle soup beans	2.53	2.65	4.53	21.5	23.3	7.73	5.78	5.45	5.71
Red Mexican beans	2.25	2.34	3.85	18.2	18.5	1.62	8.12	7.43	8.50
Peanuts									
Runner	1.55	1.57	1.27	151.0	144.0	4.64	9.80	9.40	4.08
White Spanish	1.46	1.52	3.95	150.0	158.0	5.06	8.47	8.52	0.59
Lentils	2.56	2.60	1.54	25.6	26.8	4.48	6.93	6 69	3.46
		Average	*2.67*		Average	*2.97*		Average	*4.48*

[1] Values are given for one sample of each variety of legume.

stances which interfere with the microbiological assay. In the course of the preliminary work on niacin, it was difficult to obtain agreement between the two methods when determining the niacin content of Great Northern and lima beans. There was less variation among replicates determined by the microbiological method than those obtained by the chemical procedure. Therefore, the microbiological method was selected for the routine analysis for niacin. The thiochrome method was decided upon for thiamine because more samples could be analyzed in a given period of time than by the fermentation method, since the fermentometer available for this study was a small model.

TABLE 2

Riboflavin, niacin and thiamine content of dried legumes, as obtained.

PRODUCT	NO OF SAMPLES[1]	AVERAGE MOISTURE	RIBOFLAVIN		NIACIN		THIAMINE	
			Range	Average	Range	Average	Range	Average
		%	µg./gm.	µg./gm.	µg./gm.	µg./gm.	µg./gm.	µg./gm.
Red kidney beans	12	8.88	1.86–2.38	2.15	21.1–30.6	25.9	4.18– 8.60	6.37
White marrow beans	6	9.44	1.55–1.87	1.66	23.4–30.3	26.1	5.02– 8.48	6.08
Pea beans								
Robust	5	5.54	1.74–1.89	1.79	26.5–29.9	28.7	4.78– 8.46	6.30
Michelite	4	6.90	1.66–1.74	1.70	26.4–34.4	29.1	6.02– 7.70	6.56
Great Northern beans	9	6.50	1.84–2.08	1.95	16.6–21.9	19.7	7.00– 9.60	8.24
Yelloweye beans	5	6.99	1.71–1.89	1.80	21.4–26.7	24.8	6.11– 9.00	7.40
Pinto beans	5	7.06	2.16–2.39	2.23	17.7–27.5	21.8	6.42– 7.65	7.18
Lima beans								
Baby	8	7.74	1.47–2.11	1.74	20.5–26.3	22.7	3.66– 6.52	4.86
Fordhook	4	9.50	1.59–1.85	1.71	20.5–23.0	21.7	3.00– 3.93	3.57
Large flat	4	9.77	1.70–1.74	1.72	19.7–22.1	21.1	4.00– 4.78	4.35
Blackeye peas	5	6.53	2.05–3.04	2.28	24.6–29.5	27.6	5.52– 6.80	5.92
Soybeans								
Chief	5	4.08	2.98–3.61	3.29	25.8–35.6	29.3	7.06–11.20	9.52
Richland	6	4.62	3.02–3.58	3.24	24.4–34.8	28.2	7.05–13.80	10.36
Manchu	5	5.30	2.98–3.92	3.32	19.3–37.0	24.6	6.24– 9.95	8.03
Illini	4	3.64	3.11–3.59	3.24	23.4–28.0	25.3	9.17–11.10	9.88
Bansei	5	6.11	3.01–4.12	3.45	26.0–35.3	29.2	7.41– 9.88	8.25
Split peas								
Yellow	5	8.62	1.52–1.67	1.60	28.4–33.9	31.5	6.37– 7.11	6.92
Green	5	8.25	1.64–1.78	1.72	33.3–36.9	35.8	6.62– 8.34	7.56
White kidney beans	2	6.54	1.93–1.98	1.96	31.2–33.7	32.5	6.34– 6.84	6.59
Cranberry beans	2	6.24	2.39–2.58	2.49	24.4–24.8	24.6	5.51– 5.61	5.56
Pink beans	3	5.56	2.39–2.44	2.42	19.3–20.1	19.7	8.10– 9.05	8.68
Black turtle soup beans	2	7.20	2.53–2.54	2.54	20.7–21.5	21.1	5.70– 5.78	5.74
Red Mexican beans	2	6.73	2.25–2.34	2.30	17.9–18.2	18.1	8.12– 8.40	8.26
Peanuts								
Runner	5	2.63	1.55–1.92	1.65	151.0–167.0	157.4	7.18–10.97	9.05
White Spanish	3	2.93	1.46–1.73	1.64	128.0–158.0	136.3	7.32– 8.47	7.86
Lentils	3	9.47	2.56–2.64	2.60	22.9–25.6	24.6	6.15– 7.67	6.92

[1] Each sample was obtained from a different source.

The results of the study of the riboflavin, niacin and thiamine content of leguminous seeds conducted by these methods are presented in table 2. In general, the values agree well with much of the data appearing in the literature. The average riboflavin content of legumes is rather consistent, ranging from 1.6 μg./gm. for yellow split peas and peanuts to 3.3 for soybeans. In the case of niacin there is little variation except for peanuts. Most legumes average between 18.1 μg./gm. for Red Mexican beans and 35.8 for green split peas. Peanuts are an outstanding exception, being one of the most potent natural sources of this vitamin, averaging 136 and 157 μg./gm. for white Spanish and runner peanuts, respectively. The average thiamine content of legumes falls within the range of 3.6 and 10.4 μg./gm. Lima beans of the Fordhook variety contain the least and soybeans the most.

No striking varietal differences are found, certainly not among the varieties of pea beans and lima beans. The greatest variation occurs in the niacin content of soybeans, split peas and peanuts.

From the results of this study it can be concluded that legumes are an excellent source of thiamine, and a good source of niacin and riboflavin. With a few exceptions, the greatest amount of all three vitamins is found in the soybean, thus substantiating its high status in the list of foods.

SUMMARY

A study has been made of the riboflavin, niacin and thiamine content of dried leguminous seeds. Seventeen varieties of legumes were analyzed. Two different methods were used for determining each vitamin in one sample of every variety of legume. Excellent agreement was found in all cases. The routine methods used were the fluorometric method for riboflavin, the microbiological method for niacin and the thiochrome method for thiamine.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. E. V. Hardenburg, Professor of Vegetable Crops and Dr. W. H. Burkholder, Professor of Plant Pathology, Cornell University, and to the many individuals and companies who so generously contributed samples. We are also indebted to the Poultry Department of Cornell University for the use of their facilities in carrying out this investigation.

LITERATURE CITED

BOOHER, L. E., AND E. R. HARTZLER 1939 The vitamin B_1 content of foods in terms of crystalline thiamine. U. S. Dept. of Agriculture Tech. Bull. 707.

BOOHER, L. E., E. R. HARTZLER AND E. M. HEWSTON 1942 A compilation of the vitamin values of foods in relation to processing and other variants. U. S. Dept. of Agriculture Circ. 638.

BURKHOLDER, P. R. 1943 Vitamins in edible soybeans. Science, vol. 98, p. 188.

CHELDELIN, V. H., M. A. EPPRIGHT, E. E. SNELL AND B. M. GUIRARD 1942 Enzymatic liberation of B vitamins from plant and animal tissues. The University of Texas Publication, no. 4237, p. 15.

DANN, W. J., AND P. HANDLER 1941 The quantitative estimation of nicotinic acid in animal tissues. J. Biol. Chem., vol. 140, p. 201.

FIXSEN, M. A. B., AND M. H. ROSCOE 1938 Tables of the vitamin content of human and animal foods. Nut. Abst. and Rev., vol. 7, p. 823.

HALVERSON, J. O., AND F. W. SHERWOOD 1940 The vitamin A activity and the vitamin B_1 content of soybeans and cowpeas. J. Agr. Res., vol. 60, p. 141.

HENNESSY, D. J. 1942 A standard thiochrome assay for the determination of thiamine in cereal products. Cereal Chemists' Bull., vol. 2, no. 2, p. 1.

HIGGINS, B. B., K. T. HOLLEY, T. A. PICKETT AND C. D. WHEELER 1941 Thiamine chloride and nicotinic acid content of peanuts and peanut products. Georgia Agr. Exp. Sta., Bull. 213, p. 11.

HODSON, A. Z., AND L. C. NORRIS 1939 A fluorometric method for determining the riboflavin content of foodstuffs. J. Biol. Chem., vol. 131, p. 621.

ISBELL, H. 1942 Effect of p-aminobenzoic acid on the microbiological assay for nicotinic acid. J. Biol. Chem., vol. 144, p. 567.

KELLY, E., K. S. DIETRICH AND T. PORTER 1940 Vitamin B_1 content of eight varieties of beans grown in two localities in Michigan. Food Research, vol. 5, p. 253.

MUNSELL, H. E. 1940 Vitamins and their occurrence in foods. Milbank Memorial Fund Quarterly, vol. 18, p. 311.

——— 1942 Riboflavin content of some common foods. Food Research, vol. 7, p. 85.

——— 1943 The vitamin A, vitamin B_1 (thiamine), vitamin C (ascorbic acid) and riboflavin content of common foods. Milbank Memorial Fund Quarterly, vol. 21, p. 102.

RUSSELL, W. C., M. W. TAYLOR AND J. F. BEUK 1943 The nicotinic acid content of common fruits and vegetables as prepared for human consumption. J. Nutrition, vol. 25, p. 275.

SCHULTZ, A. S., L. ATKIN AND C. N. FREY 1942 Determination of vitamin B_1 by yeast fermentation method. Improvements related to use of sulfite cleavage and a new fermentometer. Ind. Eng. Chem., Anal. Ed., vol. 14, p. 35.

SCOTT, M. L. 1943 Unpublished work. Cornell University, Ithaca, New York.

SHERMAN, H. C. 1941 Chemistry of Foods and Nutrition, Sixth Edition, New York. The Macmillan Company.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., vol. 11, p. 346.

SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., vol. 139, p. 675.

TEPLY, L. J., F. M. STRONG AND C. A. ELVEHJEM 1942 The distribution of nicotinic acid in foods. J. Nutrition, vol. 23, p. 417.

NUTRITIONAL STUDIES WITH THE DUCK

I. PURIFIED RATIONS FOR THE DUCK [1, 2]

D. M. HEGSTED AND F. J. STARE

Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston

THREE FIGURES

(Received for publication March 24, 1945)

Developments in experimental nutrition during the past decade have shown that the nutritional requirements of one species cannot be predicted from data available on other species and that the symptoms of a deficiency of the same nutrient in various animals may be markedly different. It is of interest, and perhaps unexpected, that the requirements of the chick for biotin (Hegsted et al., '42), eluate factor (Hogan and Parrot, '40; Hutchings et al., '41), and nicotinic acid (Briggs et al., '43), as well as the other members of the B-complex, appear to be quite similar to those of the monkey (Day, '44; Waisman and Elvehjem, '43), at least more similar than are the requirements of the rat and dog for these nutrients. Nutritional studies on many species are highly desirable, not only from the comparative standpoint, but also because they may find application in unexpected fields.

Only two avian species have received extensive study. At the present time, the nutritional requirements of the chick are fairly well defined. Studies on the turkey are not so complete, but the requirements for some of the vitamins and minerals and for protein can be given (Committee on Animal Nutrition, National Research Council, '44). Although the duck has been widely used as a host for experimental malarial infections, a search of the literature reveals only a few scattered references to the nutrition of this species. Pappenheimer and Goettsch ('34) have shown that vitamin E deficiency in the duck results in muscular dystrophy. Biotin deficiency and pantothenic acid deficiency have also been produced in the duck (Trager, '43) incidental to another study. The biotin

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

[2] Supported in part by a grant-in-aid from the John and Mary R. Markle Foundation.

deficiency was produced by the inclusion of raw egg white in the ration while the pantothenic acid deficiency was produced by feeding a heated ration. Although a heated ration of the type used is primarily low in pantothenic acid, it has been shown that for the chick it also contains insufficient amounts of several other factors (Waisman et al., '42).

As far as we are aware, no studies have been made with the duck using purified rations. We were interested in this species for studies on the relation of nutrition to malarial infection in a susceptible species and from the standpoint of a study in comparative nutrition. The studies reported in this paper define fairly well nutritional requirements of the duck and show that in general they are qualitatively similar to those of the chick.

EXPERIMENTAL[3]

Day-old white Pekin ducklings were used in all of these studies. Immediately after receipt from the hatchery, they were placed in heated cages equipped with raised-screen bottoms which were 22 inches square. From two to four ducks per cage have been used, but groups of two are preferable if growth approaches optimum. Food and water were allowed ad libitum. In most of the studies reported here, the birds received no food other than the experimental ration which was supplied as soon as the ducks arrived. In more recent work, however, they have usually been fed commercial duck pellets or chick mash mixed with water for the first 2 or 3 days. This has reduced the high mortality that occasionally occurred in the first few days.

In the first experiment the birds were fed a purified diet containing 3% liver extract (D1, table 1). This diet has been used with chicks and gives good growth in this species. The effect of additional liver extract and yeast was also tested. With groups of four ducks, the average daily gain per duck during the first 10 days was as follows: Diet D1 — 14.8 gm.; Diet D1 plus an additional 3% liver extract and 5% brewer's yeast — 17.1 gm.; and commercial chick mash — 16.0 gm. The ducks were then used for studies not pertinent to this paper, but it was apparent that good growth, although perhaps not optimum, could be obtained with Diet D1.

Another approach was made in experiment 2 by using a highly purified diet (D2, table 1) as the basal ration. This ration is fairly adequate for the rat but completely unsatisfactory for the chick. The results obtained with this diet and supplements of 10% gelatin, 3% liver extract,

[3] The synthetic vitamins were supplied by Merck and Company, Rahway, New Jersey, and the liver extract was supplied by The Wilson Laboratories, Chicago, Illinois.

0.5% arginine hydrochloride, and 20 µg. biotin per 100 gm. in various combinations are shown in figure 1. As for the chick, Diet D2 is completely inadequate for growth in the duckling. Biotin, liver extract, and gelatin all appear to be beneficial. Apparently arginine at 0.5% does not completely replace gelatin. In further studies in which the effect of additional casein was compared with gelatin, Diet D2 was supplemented with liver extract and biotin (Diet D4). The average weight in grams after 20 days on this diet (D4) and with supplements of casein and gelatin was as follows: D4 — 390; D4 plus 10% casein — 602; D4 plus 10% gelatin — 666. It thus appears that gelatin is superior to

TABLE 1

Composition of diets.

CONSTITUENT	D1	D2	D3	D4
	%	%	%	%
Sucrose	55	70	60	66
Casein, SMA, "vitamin-free"	20	18	18	18
Salts IV [1]	5	5	5	5
Corn oil	5	4	4	4
CaHPO4	1	1	1	1
Cod liver oil	1	2	2	2
Gelatin	7		10	
Calcium gluconate	2			
Cellu flour	1			
Liver extract [2]	3			4
Choline chloride	0.3	0.3	0.3	0.3
Vitamin mixture [3]	+	+	+	+
Biotin, 15 µg. per 100 gm.	+			+

[1] Hegsted, D. Mark. R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., vol. 138, p. 459.

[2] Wilson's Fraction "L."

[3] A mixture supplying 400 µg. thiamine hydrochloride, 400 µg. pyridoxine hydrochloride, 800 µg. riboflavin, 1.5 mg. pantothenic acid, 3.0 mg. nicotinic acid, and 100 µg. 2-methyl-1, 4-napthoquinone and 10 mg. α tocopherol per 100 gm. of ration.

casein in supplementing the ration, and certainly amino acids in addition to those supplied by 18% casein are required. If the amino acid composition is satisfactory, however, 20% protein is sufficient since the duck pellets which give excellent growth contain only 20.3% crude protein.

The need for biotin and a factor in liver extract is more clearly shown in figure 2. The basal diet used in this experiment was the highly purified Diet D2 plus 10% gelatin and is given as Diet D3 in table 1. The liver extract used in this experiment was extracted three times with boiling absolute alcohol to remove biotin. Practically no growth was

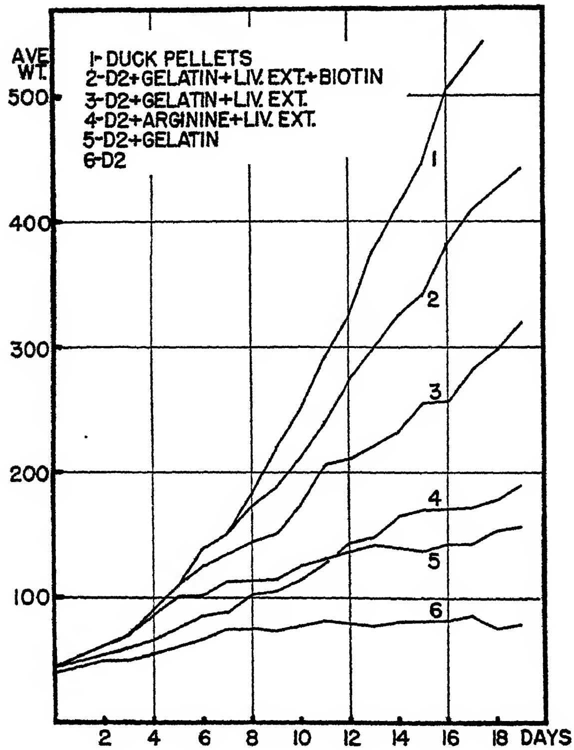


Fig. 1 The growth curves of ducks on the purified diet (D2) only and with various supplements compared to duck pellets. The supplements supplied 10% gelatin, 3% liver extract (Wilson's fraction "L"), 0.5% arginine hydrochloride, and 20 μg. biotin per 100 gm.

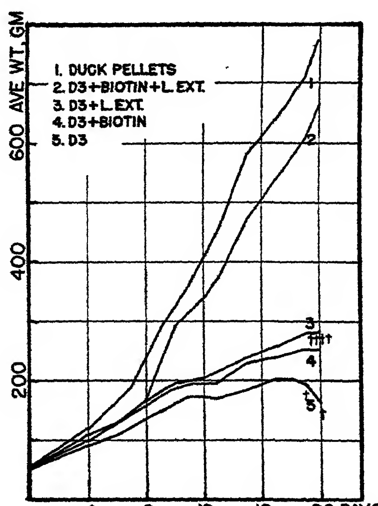


Fig. 2 Growth curves of ducks on Diet D3 plus 3% liver extract and 20 μg. biotin per 100 gm. and these supplements together. The liver extract was previously extracted with absolute

obtained with supplements of either biotin or liver extract alone, but the combination gave good growth. As in the previous experiment, the growth is still somewhat less than is obtained with a good commercial ration. The deficiency symptoms in the groups lacking biotin and liver factor were not remarkable other than the very poor growth. No signs of dermatitis were seen in the biotin deficient birds. Those lacking the liver factor appeared to develop a slight spastic paralysis after the first few days; however they improved spontaneously.

TABLE 2

The effect of various supplements on food consumption and utilization.

DIET [1]	TIME	GAIN IN WEIGHT	FOOD INTAKE PER GM. GAIN	GAIN PER GM. FOOD
	days	*gm./day*	*gm.*	*gm.*
Diet II	9 [2]	2.1	8.5	0.12
Diet II + 10 gm. gelatin	9	4.7	7.9	0.13
Diet II + 0.5 gm. arginine + 3 gm. liver extract	9	10.0	3.0	0.33
Diet II + 10 gm. gelatin + 3 gm. liver extract	9	13.8	2.8	0.35
Diet II + 10 gm. gelatin + 3 gm. liver extract + 40 µg. biotin	9	24.9	2.35	0.43
Duck pellets	9	33.4	1.80	0.55
Diet III	15 [3]	7.8	6.9	0.15
Diet III + 40 µg. biotin	15	9.5	4.0	0.25
Diet III + 3 gm. liver extract	15	12.1	3.36	0.30
Diet III + 40 µg. biotin + 3 gm. liver extract	15	33.5	2.34	0.43
Chick mash	15	35.2	2.23	0.45
Duck pellets	15	41.0	2.05	0.49

[1] Supplements given as additions per 100 gm. of ration.
[2] Experiment 2. From fifth to fourteenth day.
[3] Experiment 3. From second to seventeenth day.

The effect of the various supplements in the experiments with Diets D2 and D3 is clearly shown in table 2 in the data on food consumption. On the basal diets the food was only 12 to 15% efficient (gain in weight per gram of food), while the duck pellets were 50% efficient. As was found by weight measurements, the purified ration approaches but does not equal the results obtained on the commercial rations.

The factors required by the duck which are present in liver extract are probably the same as those required by the chick. It has been shown that they are present in an eluate prepared by the treatment of the liver extract with fuller's earth at pH 3 and elution with dilute ammonia. A concentrate of "L. casei factor"[4] was tried curatively as shown in

[4] Kindly supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, New York. This preparation contained 0.85 mg. of L. casei factor in 17 mg. of solids according to Dr. Stokstad.

figure 3. The basal diet in this experiment was D3 plus 40 μg. of biotin per 100 gm. of ration, and the curve represents the average weight of three ducks in the group. It is apparent that the oral supplements of this concentrate resulted in a marked growth response; larger amounts at the beginning of supplementation might have made this more marked. Ducks deficient in the eluate factor show a severe anemia although this is much less consistent than is the growth failure. Hemoglobin values of 4 and 5 gm. per cent and hematocrit reading below 10% are observed in severe deficiency.

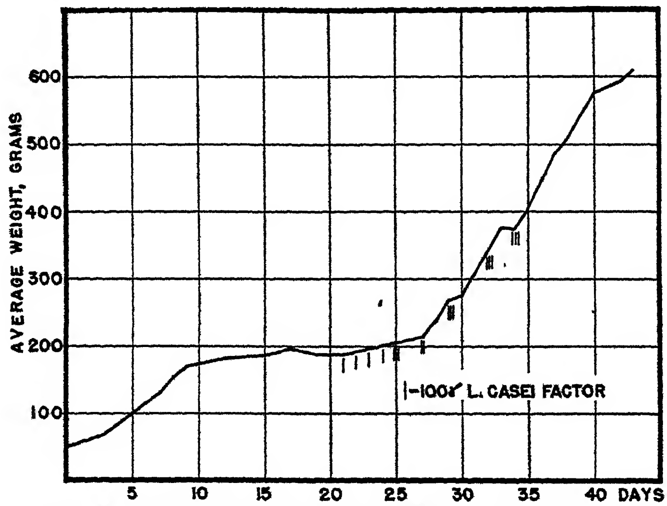


Fig. 3 The effect of supplements of L. casei factor on ration D3 plus biotin.

DISCUSSION

The studies reported in this paper indicate that the nutritional requirements of the duck are qualitatively similar to those of the chick. The duck has a very rapid rate of growth which appears to make it very susceptible to nutritional deficiencies. In studies on specific deficiencies to be reported later, it has been found that deficiencies of most of the vitamins so far studied may be easily produced by the omission of the appropriate vitamin from Diet D3 plus liver extract and biotin. In practically all instances the deficiency is clearly evident within a week after the experiment is started.

The very high efficiency of food utilization noted in these studies has been found previously. Titus ('39) gives the pounds of feed required to produce a live weight of one pound in various birds as follows: White Leghorns. 3.18; Rhode Island Red chickens, 2.53; turkeys, 2.20; and

White Pekin ducks, 2.01. It will be of interest to determine whether or not this high efficiency is associated with unusual (high or low) vitamin requirements.

Certain disadvantages of the duck as an experimental animal are also apparent. Considerable variation within a group is noted and is at least as great if not greater than is found with chicks. This together with the larger amounts of purified ration consumed, greater space requirements as compared with the chick, and the fact that it is a messy animal to work with should be recognized.

Good growth on purified rations may be obtained with the duck, but as with the rat and the chick, it falls somewhat short of that resulting from the feeding of good commercial rations. With regard to the factors in liver extract required by the duck, it is apparent that they are similar if not identical to those required by the chick. Pfeffner et al. ('43) found that vitamin B_c was the only critical material supplied by such preparations on their purified chick diet. However, Briggs et al. ('44) state that the eluate factors are multiple, one factor being required primarily for growth and another for feather development, and suggest that both of these factors may be distinct from vitamin B_c. The ducks in these studies which were fed the purified rations definitely did not show as good feather development as those fed the crude duck pellets. However, this observation is complicated by the fact that the purified rations are composed largely of sucrose and are sticky when wet. The ducks always succeed in getting considerable of this sticky ration on them, especially around the head and neck. This may be somewhat irritating and in any event makes a classification of feather development difficult. Some feather pulling in these animals has also been noted.

It has been shown previously that the high arginine and glycine requirement of the chick is due chiefly to the amino acids required for feather formation (Hegsted et al., '41). Although a requirement of the duck for glycine has not been shown, the requirement for additional arginine, above that supplied by 18% casein, is undoubtedly due to the rapid rate of growth since feather formation is very slight during the first few weeks of growth. From a comparative standpoint, it is also interesting that the birds deficient in biotin show no external symptoms comparable to the dermatitis found in the biotin deficient chick (Hegsted et al., '40).

SUMMARY

1. The duck is an extremely rapidly growing animal and on an adequate ration gains approximately 40 gm. a day during the first 3 weeks of life with an efficiency of food utilization of 50%.

2. Good growth, but somewhat less than optimum, occurs on purified diets containing only liver extract as the crude material. The factor in liver extract is similar or identical to the eluate factor or factors required by the chick. As in the chick, a deficiency results in an anemia.

3. The duck is similar to the chick in that additional amino acids above those supplied by 18% casein are required for good growth. Gelatin is superior to casein as a source of these amino acids.

4. Biotin is required by the duck. No symptoms other than very poor growth were noted in this deficiency.

LITERATURE CITED

BRIGGS, G. M., JR., T. D. LUCKEY, C. A. ELVEHJEM AND E. B. HART 1944 Further studies on vitamins B_{10} and B_{11} and their relation to "folic acid" activity. J. Biol. Chem., vol. 153, p. 423.

BRIGGS, G. M., JR., T. D. LUCKEY, L. J. TEPLY, C. A. ELVEHJEM AND E. B. HART 1943 Studies on nicotinic acid deficiency in the chick. J. Biol. Chem., vol. 148, p. 517.

DAY, P. L. 1944 The nutritional requirements of primates other than man. Vitamins and Hormones, Vol. II, Academic Press, Inc., New York.

HEGSTED, D. M., G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1941 The role of arginine and glycine in chick nutrition. J. Biol. Chem., vol. 140, p. 191.

HEGSTED, D. M., R. C. MILLS, G. M. BRIGGS, JR., C. A. ELVEHJEM AND E. B. HART 1942 Biotin in chick nutrition. J. Nutrition, vol. 23, p. 175.

HEGSTED, D. M., J. J. OLESON, R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1940 Studies on a dermatitis in chicks distinct from pantothenic acid deficiency. J. Nutrition, vol. 20, p. 599.

HOGAN, A. G., AND E. M. PARROTT 1940 Anemia in chicks caused by a vitamin deficiency. J. Biol Chem., vol. 132, p. 507.

HUTCHINGS, B. L., N. BOHONOS, D. M. HEGSTED, C. A. ELVEHJEM AND W. H. PETERSON 1941 Relation of a growth factor required by Lactobacillus casei ε to the nutrition of the chick. J. Biol. Chem., vol. 140, p. 681.

NATIONAL RESEARCH COUNCIL, COMMITTEE ON ANIMAL NUTRITION, June, 1944 Recommended nutrient allowances for domestic animals. No. 1 Recommended nutrient allowances for poultry.

PAPPENHEIMER, A. M., AND M. GOETTSCH 1934 Nutritional myopathy in ducklings. J. Exp. Med., vol. 59, p. 35.

PFEFFNER, J. J., S. B. BINKLEY, E. S. BLOOM, R. A. BROWN, O. D. BIRD, A. D. EMMETT, A. G. HOGAN AND B. L. O'DELL 1943 Isolation of the antianemic factor (vitamin B_c) in crystalline form from liver. Science, vol. 97, p. 404.

TITUS, H. W. 1939 Practical nutritive requirements of poultry. United States Department of Agriculture, Yearbook of Agriculture, p. 787.

TRAGER, W. 1943 The influence of biotin upon the susceptibility to malaria. J. Exp. Med., vol. 77, p. 557.

WAISMAN, H. A., AND C. A. ELVEHJEM 1943 The role of biotin and "folic acid" in the nutrition of the rhesus monkey. J. Nutrition, vol. 26, p. 361.

WAISMAN, H. A., R. C. MILLS AND C. A. ELVEHJEM 1942 Factors required by chicks maintained on a heated diet. J. Nutrition, vol. 24, p. 187.

STUDIES ON THE REQUIREMENTS OF THE MONKEY FOR RIBOFLAVIN AND A NEW FACTOR FOUND IN LIVER[1]

JACK M. COOPERMAN, HARRY A. WAISMAN, KEITH B. McCALL
AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

THREE FIGURES

(Received for publication April 2, 1945)

Recent reports from this laboratory have demonstrated the value of the monkey as an experimental animal in nutritional research. Waisman, Rasmussen, Elvehjem and Clark ('43) described a purified diet which is capable of maintaining monkeys in excellent health for extended periods of time, and the use of this diet has made it possible to study uncomplicated vitamin deficiencies. Studies on "folic acid" (Waisman and Elvehjem, '43), biotin (Waisman, McCall and Elvehjem, '45) and thiamine (Waisman and McCall, '44) deficiencies have already been reported. A preliminary report has been made on riboflavin studies with the monkey (Waisman, '44) and in this paper we wish to report further observations on this deficiency.

EXPERIMENTAL

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The basal diet (M-2) consisting (in %) of sucrose 73, Labco casein 18, salts IV 4, cod liver oil 3 and corn oil 2, was fed ad libitum. The following vitamin supplement was given daily: thiamine hydrochloride 1 mg., pyridoxine hydrochloride 1 mg., calcium pantothenate 3 mg., nicotinic acid 5 mg., choline chloride 25 mg., para-aminobenzoic acid 50 mg., i-inositol 50 mg., biotin 20 µg., and ascorbic acid 25 mg. In addition a norite eluate concentrate prepared according to Hutchings et al. ('41) was fed equivalent to 5 gm. of "solubilized liver residue" per animal per day.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Foundation for Infantile Paralysis, Inc., New York, and the Commercial Solvents Corp., Terre Haute, Indiana.

We are indebted to The Wilson Laboratories, Chicago, Illinois, for the various liver products and to Merck and Co., Inc., Rahway, New Jersey, for the synthetic vitamins.

The basal diet was analyzed for riboflavin by both the microbiological method of Snell and Strong ('39) as modified by Strong and Carpenter ('42) and the fluorometric method (Andrews, '43) and found to contain 0.3 μg. per gram. Since the results of both these methods checked very well, the fluorometric method was used in subsequent analyses because it was more convenient. The norite eluate concentrate contained between 1 and 2 μg. of riboflavin per gram equivalent of the original solubilized liver residue. The maximum amount of riboflavin which each animal received from this source each day was therefore 10 μg.

As previously demonstrated (Waisman, '44) the monkeys developed a "freckled type" of dermatitis in 6 to 8 weeks after being placed on the basal diet. Soon thereafter large areas of the body became covered with dark scabs which promptly regressed when riboflavin was administered. Among the other noticeable changes in the more severe cases were incoordination in the use of the limbs, faulty grasping reflex, impaired vision and a scanty hair coat. In two cases graying of the hair was observed.

Hemoglobin determinations, red blood cell and white blood cell counts were made on blood drawn from the marginal vein of the ear. Hemoglobin was determined in the Evelyn photoelectric colorimeter and red and white cell counts were made in the usual manner.

The hemoglobin level of the blood and the red cell count showed a decrease about a week after the onset of the dermatitis, and the white blood cell counts decreased to a lesser extent. The hemoglobin and red blood cell counts reached the lowest level about 5 weeks later. When riboflavin was not administered the monkeys eventually died.

In table 1 are shown the blood values for some of the monkeys before the onset of the deficiency, the lowest values at the height of the deficiency, and the levels reached after 6 weeks of riboflavin therapy. It is evident that a definite anemia appeared as part of the syndrome of riboflavin deficiency. The hemoglobin and red blood cells decreased in approximately the same ratio indicating a hypochromic, normocytic type of anemia. The white cell counts responded to riboflavin therapy to a lesser extent than did the hemoglobin and red cell counts.

These changes are clearly indicated in figure 1 which shows the blood picture of a typical monkey. It is interesting to note that the decrease in weight is not as sharp as in "folic acid" deficient monkeys (Waisman and Elvehjem, '43). This may be partly explained by the fact that the monkeys never developed anorexia during the course of the deficiency. There is, however, a definite weight response when riboflavin

One hundred micrograms per day of riboflavin was found to be the minimal amount which brought about the regression of the symptoms in all the monkeys. Fifty micrograms had variable effects, being effective in the milder cases but ineffective in more advanced cases. One hundred micrograms generally brought about the remission of the dermatitis in 10–14 days, although in some cases only 5 days elapsed before remission occurred.

In several cases it required a week of riboflavin therapy before an increase in hemoglobin and red cells was apparent but after the initial lag the increase was rapid.

TABLE 1

Values for the blood constituents of monkeys before, during and after riboflavin deficiency.

MONKEY NO.	HEMOGLOBIN			RED BLOOD CELLS			WHITE BLOOD CELLS		
	Before deficiency	During deficiency	After therapy	Before deficiency	During deficiency	After therapy	Before deficiency	During deficiency	After therapy
53	13.4	4.9	10.73	5.5	2.0	3.10	11.0	8.9	10.6
81	13.0	7.72	12.76	6.1	3.02	3.25	11.0	6.09	9.8
82	12.9	8.0	12.41	6.09	1.7	3.80	13.8	11.8	10.0
125	13.11	9.22	12.06	4.30	2.0	3.30	14.6	8.0	12.6
6	13.38	8.63	12.14	4.28	2.7	3.20	14.6	6.2	12.8
13	13.89	5.07	11.37	4.96	1.89	3.70	15.6	11.0	12.6
128	13.50	8.99	12.06	4.52	3.60	3.20	14.7	12.4	9.6
135	13.50	5.54	12.14	4.10	1.90	3.16	11.4	4.6	10.4
166	13.89	6.76	12.76	3.95	2.0	3.25	14.2	9.2	10.0
164	14.28	7.72	11.48	4.30	2.2	3.30	10.0	6.8	9.2

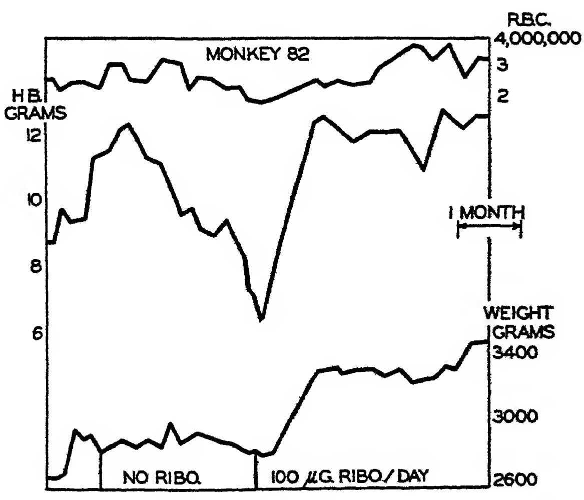


Fig. 1 Effect of riboflavin deficiency on weight, hemoglobin and red blood cell level of typical monkey, and the response to riboflavin therapy.

The deficient monkeys were examined with a slit lamp for corneal vascularization, and in none of them were any signs of corneal vascularization or opacity evident. In fact some of the control monkeys on a complete ration showed more vascularization. In no case did any cheilosis or tongue involvement appear.

Liver studies

Three of the deficient monkeys were sacrificed and upon autopsy no gross pathology except yellow livers was evident. The livers were removed, dried, and analyzed for fat. At the same time, livers from two monkeys which had died of acute "folic acid" deficiency were analyzed for fat for comparison. The riboflavin content of the various livers was also determined, and all of the data are presented in table 2.

TABLE 2

The fat and riboflavin content of the livers of riboflavin deficient and non-riboflavin deficient monkeys.

	RIBOFLAVIN DEFICIENT			NON-RIBOFLAVIN DEFICIENT	
MONKEY NO.	57	80	64	32	63
Fat (% dry basis)	26.22	47.01	42.5	23.96	12.75
Riboflavin (mg./100 gm. dry basis)	3.62	3.27	2.02	8.00	9.00

It will be noted that a distinct fatty liver condition existed in the riboflavin deficient monkeys. Sebrell and Onstott ('38) also noted yellow livers in riboflavin deficient dogs. Other workers have claimed that this is due to inanition rather than riboflavin deficiency. The results in the case of the monkey are interesting since riboflavin deficient monkeys do not develop anorexia. On the other hand "folic acid" deficient monkeys do show a reduced food intake and yet the liver fat values are distinctly lower than the values for riboflavin deficient monkeys. The reason that the liver fat in monkey 57 is not as high as in the other two monkeys is undoubtedly due to the fact that this animal was on the deficient diet for a shorter period of time.

The differences in riboflavin content of the livers of riboflavin deficient and non-riboflavin deficient monkeys are noteworthy. The value for the deficient animals drops to approximately one-third of the value for non-deficient animals. This corresponds well with the work of Kuhn, Kaltschmitt and Wagner-Jauregg ('35) and Vivanco ('35) in which they showed that the body conserves its store of riboflavin, and that

even in rats that die for lack of this vitamin, the amount in the liver, kidney and heart is still about one-third of the normal level.

Excretion studies

Four of the monkeys were placed in metabolism cages which were similar to the regular cages except that they are equipped with a funnel-shaped, metal bottom for the collection of urine. A fine screen was placed below the regular floor of the cage in order to collect the feces. Sheets of tin were placed on the screen floors of the metabolism cages under the food cups to prevent food from falling into the urine. Whenever the monkeys showed diarrhea the urine samples were disregarded. The urines were stored in brown bottles with measured amounts of glacial acetic acid and toluene added and placed in the cold room until they were assayed. The feces were collected and dried at 45°C. for 24 hours in a vacuum oven. After drying the feces were placed in brown bottles and stored in the cold room until they were assayed. The fluorometric method of assay was employed.

The riboflavin content of the feces was expressed as micrograms per dry gram rather than total weight of feces since it was not possible to remove quantitatively all the feces from the screen floor of the cage. However, the values obtained remained constant regardless of the intake or state of deficiency of the animals, the average value being 30 μg. of riboflavin per gram of dry feces. Since the daily total excretion via the feces exceeded in most cases the daily intake of riboflavin, it seems apparent that the greater part of the riboflavin in the feces is dependent upon the synthesis of this vitamin by the intestinal flora. Apparently most of the riboflavin synthesized by the flora is not available to the monkey.

Monkey 53. This animal had been on the deficient diet until dermatitis and the abnormal blood picture appeared; 100 μg. of riboflavin were then given daily for 20 days. At the end of this period, the dermatitis had disappeared and the hemoglobin and red cell content of the blood had increased. The monkey was then put on a riboflavin deficient diet and the urine and feces collected during 24-hour periods. The only source of riboflavin was the basal ration from which the monkey received an average of 40 μg. per day. For 2 weeks the daily output of riboflavin in the urine was relatively high, averaging 60–90% of the intake. After the third week the average daily output was 12% of the intake and remained at this level until a single dose of riboflavin was given. At this time the excretion increased to about 50% of the intake. Shortly after the ad-

ministration of the single dose the excretion fell to 12% of the intake and symptoms of riboflavin deficiency began to appear.

Monkey 82. Monkey 82 was on a regimen similar to that of monkey 53. It had been on a deficient diet and after the symptoms of riboflavin deficiency appeared, it was given 500 μg. of riboflavin daily for 15 days. At the end of this period the dermatitis had cleared up and the blood picture approached normal. Riboflavin was then discontinued and excretion studies started 3 days later. For the first 5 weeks the daily output of riboflavin by way of the urine averaged 60–95% of the intake. By the seventh week the 24-hour riboflavin output averaged 15% of the intake. It remained at a level of 10–15% until a test dose of riboflavin was given. At this point the output rose to 60% of the dietary intake.

The results of these two experiments agree well with recent studies by two groups of workers. Williams et al. ('43) found that the daily output of riboflavin dropped to a level of 14% of the intake during the depletion period in human subjects.

Keys and coworkers ('44) working with young men found that during the depletion period the 24-hour output dropped to 12% of the intake. When a test dose was administered the output rose to 54% of the intake.

It is also significant to note that none of the above workers found vascularization of the cornea or cheilosis among the deficient men.

Monkey 64. Monkey 64 had been on the deficient diet and after the typical symptoms appeared, therapy of 50 μg. per day was started. Excretion studies were started 12 days after therapy was instituted. The average daily output varied between 40–60% of the intake for a 3-month period.

Monkey 81. Monkey 81 was on a regimen similar to that of monkey 64. It too was put on metabolism studies 2 days after riboflavin therapy was instituted. Previous to that it had been in the deficient state. This monkey was also receiving a daily supplement of 50 μg. of riboflavin a day in addition to what it received in the basal ration. For the entire period the daily output varied between 30–80%.

From the above two studies it would appear that the deficient animal does not retain all the ingested riboflavin.

Feder, Lewis and Alden ('44) have suggested that when the excretion of riboflavin is expressed in micrograms per milliliter of urine, a more accurate estimation of the body's supply of riboflavin is possible. They assumed that an excretion below 3 μg. per milliliter shows a state of deficiency. In our study an attempt was made to express excretions on this

basis but results were variable. A better correlation was obtained when the results were expressed as total riboflavin output for a 24-hour period. The discrepancies might be partially explained by the fact that frequently the monkeys spilled their drinking water into the urine collection jars. This would make any results based on micrograms per milliliter of urine meaningless.

A summary of the excretion studies is presented in table 3.

TABLE 3

Urinary excretion of riboflavin.

WEEK	AVERAGE DAILY RIBOFLAVIN EXCRETION IN MICROGRAMS				AVERAGE DAILY EXCRETION AS PER CENT OF INTAKE			
	Monkey				Monkey			
	53	82	64	81	53	82	64	81
1	36	38	52.2	72	90	95	58	80
2	33.6	32	43.3	43.3	84	80	47	47
3	28	36	36	55.8	70	90	40	62
4	6	26.8	48.6	67.5	15	67	54	75
5	4.8	24	55.8	45	12	60	62	50
6	5.6	6	45	36	14	15	50	50
7	5.6	7.2	40.5	33.3	14	18	45	37
8	4.8	5.6	43.2	18	12	14	48	20
9	4.8	6	45	43.3	12	15	50	47
10	4.8	5.6	54	64.8	12	14	60	72
11	75.6	351			54	65		
Total daily intake of riboflavin	40 [1]	40 [2]	90	90				

[1] Increased to 140 at 11th week.
[2] Increased to 540 at 11th week.

Studies on a new factor for the monkey

As has already been demonstrated, a part of the syndrome of riboflavin deficiency in monkeys is the occurrence of a hypochromic, normocytic anemia. Upon administration of riboflavin there is an increase in hemoglobin, red cell and white cell content of the blood. However, a plateau is soon reached in both the weight and blood responses. If the blood picture had reached normal values this phenomenon would be expected. However, the hemoglobin content did not reach accepted normal levels. A survey of the literature revealed conflicting data on the exact normal blood picture of the rhesus monkey. Shukers et al. ('38) reported a study upon nineteen monkeys comparing their results with previously published works and their values are as follows: hemoglobin 10.0–13.5 gm. %; red blood cells 4.6–5.8 millions per cubic millimeter;

white blood cells 9.7–20.5 thousands per cubic millimeter. Two Indian workers, Rao and Rao ('40), studying macacus sinicus reported a large range in the normal blood picture of the monkey.

Since the diets which the various workers used and the methods of determination varied greatly, it is difficult to evaluate the results. Studies in our laboratory on normal, healthy, young monkeys receiving 3% liver powder in addition to the basal ration showed a normal range as follows: hemoglobin 14–15.5 gm. %; red blood cells 4–5.5 millions per cubic millimeter; white blood cells 10–25 thousands per cubic millimeter. Referring again to table 1 we see that the plateau occurs at a level well below that which can be considered normal. This plateau occurred after treatment of every deficient monkey, whether the daily therapeutic dose was 100, 500 μg, or 1 mg. of riboflavin.

These results indicated that other factors were necessary to produce a normal growth and concentration of blood constituents. Therefore, monkey 125, after reaching this plateau, was placed on the basal ration M-2 plus 3% 1:20 liver extract, the liver powder being added at the expense of the dry part of the ration. After 3 months on this ration there was no apparent improvement.

The monkey was then placed on the M-2 basal ration for 2 weeks, after which it was changed to M-2 basal plus 3% whole liver powder. Within a week there was a perceptible increase both in weight and blood constituents. Within 3 weeks the blood hemoglobin level rose from 12 gm. to 14.5 gm. per 100 ml. and the red cell count increased proportionately. A sharp increase in weight also occurred.

Two other monkeys, numbers 53 and 128, which were at the plateau stage after recovering from a riboflavin deficiency were then placed on the M-2 basal plus 3% whole liver substance. In monkey 128 the hemoglobin increased from a level of 12.4 to 14.7 gm. %. The red cell count rose from 3.4 to 4.8 millions per cubic millimeter.

After 9 weeks on this regimen, the animal was returned to the M-2 basal without the liver. The weight continued to increase for about 3 weeks at which time it showed a plateau. The blood picture fell more rapidly than the weight and the hemoglobin value returned to 12.3 gm. % in 3 weeks.

The effect of the whole liver substance on monkey 53 is shown in figure 2. Here again a rapid and sharp increase in weight, hemoglobin and red cells content was demonstrated. It is also interesting to note the increase in the white blood cell count. This increase in the blood constituents and weights occurred in every case without exception when a monkey having reached the plateau stage was given the ration con-

taining 3% whole liver substance. In a period of 3½ months, monkey 53 gained over 2 kg. in weight. It had gained 200 gm. over a similar period when riboflavin alone was given without the liver. In 1 month's time on whole liver substance the hemoglobin increased from a level of 10.5 to 14.6 gm. %, the red cells from 3.1 to 5.1 millions per cubic millimeter, and the white cells from 11 to 16 thousands per cubic millimeter. Here again the discontinuance of the liver substance produced a plateau in both the weight and blood elements and a downward trend after a few weeks.

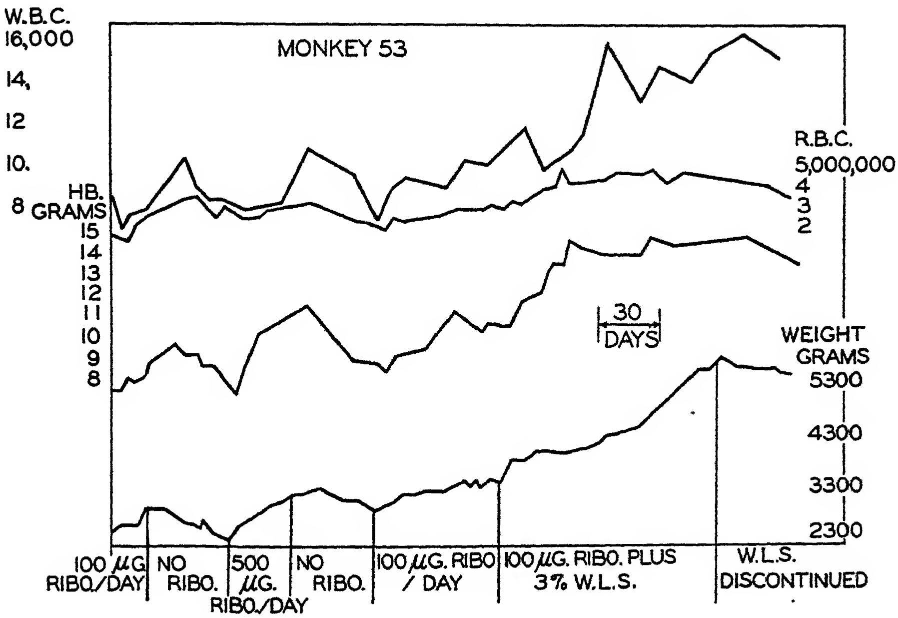


Fig. 2 Curve representing effect of whole liver powder on weight, hemoglobin, red and white cell count in typical riboflavin deficient monkey which showed a plateau following riboflavin therapy.

Scott and coworkers at Cornell ('44) reported that pyridoxine treated with hydrogen peroxide promoted growth and more particularly hemoglobin formation in chicks. Accordingly hydrogen peroxide treated pyridoxine or "pseudo-pyridoxine" was prepared by the method of Carpenter and Strong ('44) and fed to two monkeys, numbers 128 and 82, at a level equivalent to 1 mg. of the original pyridoxine per day. From figure 3 it can be seen that the preparation had no effect in monkeys 82 and 128 on growth or the blood picture over a period of 3 and 4 weeks, respectively.

Monkey 82 was then put on a regimen of the M-2 basal plus 3% 1:20 liver extract powder. From figure 3 it is apparent that this had no additional effect. Any differences may be accounted for by the fact that different batches of liver powder were used in each case.

In a report from Puerto Rico (Report of Director, '43) there appeared a note that the iron content of ordinary laboratory rations was too low for monkeys. Accordingly, two monkeys, numbers 164 and 128, were given in addition to the iron supplied by the basal ration 10 mg.

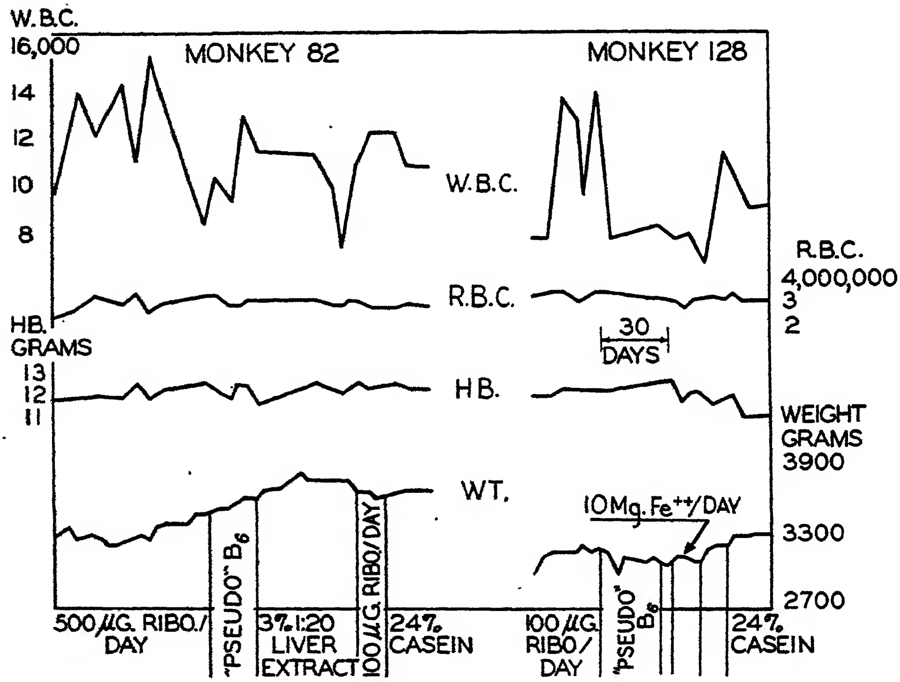


Fig. 3 Effect of various supplements on hemoglobin level, red and white blood cell count, and weight of two typical monkeys which reached the plateau stage following riboflavin therapy. Supplements added in addition to riboflavin.

of iron in the form of hydrated ferrous ammonium sulfate. From figure 3 we see that the additional iron had no hemoglobin building or growth promoting properties. A similar effect was noticed with monkey 164.

In order to determine if the protein level of the ration was adequate for optimum growth and hemoglobin production, monkeys 128 and 82 were given a ration containing 24% casein, the extra casein being added at the expense of the sucrose. It is evident from figure 3 that adding extra protein had no effect either on the blood picture or weight response.

Three monkeys, numbers 82, 128 and 164, were fed the M-2 basal plus 3% extracted liver residue, the fraction left when 1:20 liver extract is made from whole liver powder. However, after 4 weeks on this ration none of the animals responded in any way.

One monkey was maintained on a "complete" purified ration M-2 plus all the known vitamins and the norite eluate concentrate. After 18 months on this ration the monkey suddenly lost weight and was saved through the use of whole liver powder therapy.

DISCUSSION

Classical studies have resulted in the association of riboflavin deficiency with cheilosis and corneal vascularization and slit lamp studies of the eye are routinely used to diagnose the lack of this vitamin. Recent studies (Williams et al., '43; Keys et al., '44) have shown that this syndrome is not necessarily a part of riboflavin deficiency and have indicated that many cases showing these symptoms fail to respond to riboflavin therapy. This study indicates that cheilosis or corneal involvement was not necessarily a part of the riboflavin deficiency syndrome. However, these studies must be differentiated from chronic deficiencies more generally encountered in clinical practices.

Although no attempt was made to establish the exact riboflavin requirement of monkeys, certain calculations can be made regarding the requirement when a high sugar diet is used. It is obvious that all monkeys developed a deficiency on the basal ration supplying 40 µg. per monkey per day, or about 12 µg. per kilogram of body weight. The addition of 25 µg. failed to give a response in the deficient animals, but the addition of 50 µg., or a total intake of 90 µg. per day, cured the symptoms in the monkeys with the milder symptoms and allowed considerable excretion in the urine. Thus the requirement of a young monkey is probably 25 to 30 µg. per kilogram of body weight per day. Whether the amount for maintenance of adult monkeys is less cannot be determined from the present data, but it is entirely possible that the requirement may be as low as 15 to 20 µg. per kilogram. These figures are of the same magnitude as the riboflavin requirement of dogs as established by Spector et al. ('43).

It has become evident during the course of these studies that the synthetic ration used in these studies is not complete for the monkey. A factor (or factors) which is found in whole liver powder appears to be necessary for optimum growth and blood regeneration. A riboflavin deficiency precipitates the need for this factor (or factors). The administration of riboflavin results in a weight gain accompanied by a sig-

nificant rise of hemoglobin level and red cell count but these increases do not attain accepted normal values. Such monkeys can then be used as assay animals for the factor (or factors) in whole liver powder This factor (or factors) is evidently labile since commercial extraction procedures used to prepare 1:20 liver extract and extracted liver residue from whole liver destroy nearly all activity. Recently Day and co-workers ('45) reported that injection of highly purified preparations of the Lactobacillus casei factor resulted in a return of the total white blood cell and granulocyte counts to normal levels in vitamin M deficient monkeys. However, there was little improvement in the hemoglobin levels and no weight data were given. That our factor described above appears to be different is evident from the fact that the norite eluate concentrate used in our ration is a rich source of the Lactobacillus casei factor.

SUMMARY

Rhesus monkeys were placed on a riboflavin deficient diet and after 6 to 8 weeks a loss in weight became apparent and soon thereafter a freckled type of dermatitis appeared first on the face, then on the hands, legs and groin. Shortly after the appearance of the dermatitis the hemoglobin, red cells and to a lesser extent the white cell level showed a decrease, a hypochromic normocytic type of anemia developing. With the administration of riboflavin growth was resumed, the dermatitis regressed and the anemia showed some improvement.

Excretion studies showed that normal monkeys excrete riboflavin daily at the same range as normal humans and that on a deficient diet the daily output drops to 10–15% of the daily intake. Saturation tests fail when applied as a diagnostic tool to determine riboflavin deficiency in the monkey.

Fatty livers which cannot be explained on the basis of inanition were demonstrated in monkeys that had died of riboflavin deficiency.

The minimal daily requirement for young monkeys appears to be approximately 25 to 30 μg. per kilogram of body weight.

Shortly after the administration of riboflavin to deficient monkeys a plateau was reached in the values for their hemoglobin, red and white cells and weights below normal levels. Iron, "pseudopyridoxine," 1:20 liver powder, extracted liver residue, and increasing the casein level to 24% proved ineffective in restoring the blood picture and weight to a normal level. However, a factor (or factors) found in whole liver substance was necessary at a level of 3% of the ration for optimum growth and blood regeneration.

ACKNOWLEDGMENT

We wish to acknowledge the assistance of Arthur M. Ingebritsen for his help in the blood and excretion studies.

LITERATURE CITED

ANDREWS, J. S. 1943 Report of 1942–43 methods of analysis sub-committee of riboflavin assay. Cereal Chem., vol. XX, p. 613.

CARPENTER, L. E., AND F. M. STRONG 1944 Determination of pyridoxine and pseudo-pyridoxine. Arch. Biochem., vol. 3, p. 375.

DAY, P. L., V. MIMS, J. R. TOTTER, E. L. R. STOKSTAD, B. L. HUTCHINGS AND N. H. SLOANE 1945 The successful treatment of vitamin M deficiency in the monkey with highly purified Lactobacillus casei factor. J. Biol. Chem., vol. 157, p. 423.

FEDER, V. H., G. T. LEWIS AND H. S. ALDEN 1944 Studies on the urinary excretion of riboflavin. J. Nutrition, vol. 27, p. 347.

HUTCHINGS, B. L., N. BOHONOS AND W. H. PETERSON 1941 Growth factors for bacteria. XIII. Purification and properties of an eluate factor required by certain lactic acid bacteria. J. Biol. Chem., vol. 141, p. 521.

KEYS, A., A. F. HENSCHEL, O. MICKELSON, J. M. BROZEK AND J. H. CRAWFORD 1944 Physiological and biochemical functions in normal young men on a diet restricted in riboflavin. J. Nutrition, vol. 27, p. 165.

KUHN, R., H. KALTSCHMITT AND T. WAGNER-JAUREGG 1935 Über den Flavingehalt der Leber und Muskulatur von gesunden und B_2-avitaminotischen Ratten. Ztschr. f. physiol. Chem.. vol. 232, p. 36.

RAO, M. V. R., AND M. N. RAO 1940 Normal haematological standards in the monkey (Mamacus sinicus). Ind. J. Med. Res., vol. 24, p. 1101.

REPORT OF THE DIRECTOR, SCHOOL OF TROPICAL MEDICINE 1943 San Juan, P. R., p. 27.

SCOTT, M. L., L. C. NORRIS, G. F. HEUSER, W. F. BRUCE, H. W. COOVER, JR., W. D. BELLAMY AND I. C. GUNSALUS 1944 A new chick antianemic factor. J. Biol. Chem., vol. 154, p. 713.

SEBRELL, W. H., AND R. H. ONSTOTT 1938 Riboflavin deficiency in dogs. Pub. Health Reports, vol. 53, p. 83.

SHUKERS, C. F., W. C. LANGSTON AND P. L. DAY 1938 The normal blood picture of the young rhesus monkey. Folia Hemat., vol. 60, p. 416.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. and Eng. Chem., vol. 11, p. 346.

SPECTOR, H., A. R. MAASS, L. MICHAUD, C. A. ELVEHJEM AND E. B. HART 1943 The role of riboflavin in blood regeneration. J. Biol. Chem., vol. 150, p. 75.

STRONG, F. M., AND L. E. CARPENTER 1942 Preparation of samples for microbiological determination of riboflavin. Ind. and Eng. Chem., vol. 14, p. 909.

VIVANCO, F. 1935 Zur Flavinbilanz in Tierkörper. Naturwissensch., vol. 23, p. 306.

WAISMAN, H. A. 1944 Production of riboflavin deficiency in the monkey. Proc. Soc. Exp. Biol. and Med., vol. 55, p. 69.

WAISMAN, H. A., AND C. A. ELVEHJEM 1943 The role of biotin and "folic acid" in the nutrition of the rhesus monkey. J. Nutrition, vol. 26, p. 361.

WAISMAN, H. A., AND K. B. MCCALL 1944 A study of thiamine deficiency in the monkey (Macaca mulatta). Arch. Biochem., vol. 4, p. 265.

WAISMAN, H. A., K. B. MCCALL AND C. A. ELVEHJEM 1945 Acute and chronic biotin deficiencies in the monkey (Macaca mulatta). J. Nutrition, vol. 29, p. 1.

WAISMAN, H. A., A. F. RASMUSSEN, JR., C. A. ELVEHJEM AND P. F. CLARK 1943 Studies on the nutritional requirements of the rhesus monkey. J. Nutrition, vol. 26, p. 205.

WILLIAMS, R. D., H. L. MASON, P. L. CUSICK AND R. M. WILDER 1943 Observations on induced riboflavin deficiency and the riboflavin requirement of man. J. Nutrition,

GOITER STUDIES WITH THE RAT [1]

A. W. HALVERSON, JAMES H. SHAW AND E. B. HART
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

ONE PLATE (SIX FIGURES)

(Received for publication April 11, 1945)

Kraus and Monroe ('30) and Levine et al. ('33) have shown that goiter in the rat is fundamentally a result of iodine deficiency. Levine and co-workers have reported that the daily iodine requirement of the rat is 1–2 μg. The present study was undertaken to determine the iodine requirement of the growing rat on a synthetic ration to which all of the known dietary essentials were added. Since soybeans are low in iodine (Wilgus, et al., '41) and contain high quality proteins (Barnes and Maack, '43), soybean globulin was selected for the protein source.

EXPERIMENTAL

Four groups of rats were used in a 13-week experiment. Each group consisted of four female white rats which weighed 39–49 gm. and averaged 43.2 gm. at the beginning of the experiment. The rats were allowed to consume the ration ad libitum. Records of food consumption and of the growth rate were kept.

The low-iodine ration (Teresi, '43) consisted of: 75 parts recrystallized sucrose, 20 parts autoclaved soybean globulin, 4 parts salts, 5 parts corn oil, 0.6 part cystine, 0.1 part choline chloride. The percentage composition of the salt mixture which was prepared from reagent grade minerals, follows: $Ca_3(PO_4)_2$, 61.0; NaCl, 20.0; KCl, 10.0; $MgSO_4 \cdot 7H_2O$, 8.0; $FeCl_2 \cdot 4H_2O$, 0.73; $CuSO_4 \cdot 5H_2O$, 0.09; $MnSO_4 \cdot H_2O$, 0.09; and $ZnSO_4$, 0.09. The $Ca_3(PO_4)_2$ was purified further by dissolving it in reagent grade HCl. After the undissolved particles had been filtered off, the $Ca_3(PO_4)_2$ was precipitated by the addition of chemically pure NH_4OH to a pH of 4. The precipitated product was filtered, rinsed with absolute alcohol, and dried at 37°C. for 24 hours.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

These vitamins were added at the following levels per kilogram of ration: 10.0 mg. calcium pantothenate, 2.0 mg. thiamine hydrochloride, 10.0 mg. riboflavin, 6.0 mg. pyridoxine, and 65.0 mg. alpha tocopherol. One hundred and twenty micrograms of beta carotene in Wesson oil and 250 U.S.P. units of crystalline vitamin D in propylene glycol (Drisdol) were fed by dropper to each rat every week.

The soybean globulin contained 80.0% protein as determined by Kjeldahl analysis. The nitrogen factor, 5.7, was used to calculate the protein content. The preparation (Teresi, '43) of the globulin involved the dialysis of an aqueous NaCl extract of raw, ground soybeans. The precipitated globulin was centrifuged with alcohol and finally with ether to purify and facilitate the drying of the final product.

The rats in group 1 received the basal ration only. Those in groups 2 to 4 received, respectively, 0.5, 1.0, and 2.0 μg. of iodine per rat per day. The iodine supplement, which consisted of potassium iodide in redistilled water was given in 3 ml. amounts contained in crock cups each morning. When the iodine supplement had been consumed, an adequate volume of redistilled water was given to supply the daily water requirements of the rats.

After 13 weeks on the experimental diet the animals were sacrificed by ether anesthesia. The thyroids were removed with the aid of a binocular dissecting microscope and weighed immediately on a torsion balance. The thyroids were then fixed in Bouin's fluid. Histological examination was made after the glands had been sectioned and stained in hematoxylin and eosin.

RESULTS

The groups supplemented with potassium iodide showed no significant differences in food consumption or rate of growth from the group which received no iodine supplement.

The thyroid glands of the rats on the basal group were definitely enlarged and red. When 0.5 μg. of iodine per rat per day was given, the thyroid enlargement was greatly reduced, but the red appearance was still noticeable. The thyroids of the groups which received 1.0 and 2.0 μg. of iodine per rat per day were smaller and more normal in color. No significant differences were noted in the gross appearance of the glands from the two groups.

Table 1 shows the rat weights at the termination of the experiment, the weights of the dissected thyroid glands, and the calculated thyroid weights per 100 gm. of rat tissue. The thyroid weights represent the

combined weights of the freshly dissected thyroid and parathyroid glands.

Upon histological examination, the thyroid glands of the rats which received only the basal ration appeared completely abnormal. Acinar epithelial hypertrophy was present in large areas of the gland and no colloid was found in the acini (fig. 1). What little colloid remained

TABLE 1

Thirteen-week experiment showing effect of iodine supplements on thyroid weights.

GROUP NO.	IODINE SUPPLEMENT PER RAT PER DAY	RAT WEIGHTS	THYROID WEIGHTS	THYROID WEIGHTS PER 100.0 GM. RAT WEIGHT
			mg.	*mg.*
I	No supplement	200.0	40.8 [1]	20.4
		188.0	38.4 [1]	20.4
		235.0	67.1 [2]	28.6
		180.0	24.6 [1]	13.7
Average				20.8
II	0.5 µg. iodine	179.0	20.4 [2]	11.4
		200.0	24.0 [2]	12.0
		193.0	25.0 [1]	13.0
		206.0	27.8 [1]	13.5
Average				12.5
III	1.0 µg. iodine	201.0	19.0	9.5
		181.0	18.8	10.4
		130.0	15.0	11.5
		176.0	16.5	9.4
Average				10.2
IV	2.0 µg. iodine	191.0	19.2	10.1
		209.0	14.1	6.8
		194.0	24.1 [2]	12.4
		129.0	14.8	11.5
Average				10.2

[1] Gland of red appearance.
[2] Gland of pink appearance.

contained large numbers of necrotic nuclei (fig. 2). In other areas, a necrosis of the acinar epithelium had occurred which varied from a mild involvement (fig. 3), to a complete necrosis (fig. 4) and a disappearance of any semblance of normal structure or arrangement (fig. 5). There were fewer areas of complete necrosis in the thyroids of the rats which received 0.5 µg. of iodine per day; otherwise, they resembled the thyroids of the basal group. No normal areas were observed. The thyroid

glands of the rats which received 1.0 μg. of iodine per day showed widespread areas of early necrosis and hypertrophy with no accumulation of colloid. A few small areas of fairly normal acinar epithelium with colloid accumulation were found. However, in all cases, numerous necrotic nuclei were present in the colloid. The thyroid glands of those rats which received 2.0 μg. of iodine per day were almost normal (fig. 6). There were large areas of normal acini with distinctly flattened, cuboidal epithelium and smooth vacuolated colloid; however, a few small areas with necrotic nuclei in the colloid and mild necrosis of the epithelium were observed.

DISCUSSION

The results indicate that iodine is the determining factor in preventing the development of goiter in rats on this ration. The maximum weekly growth observed was 24 gm. per rat during the fifth week of the experiment; however, the overall rate of growth was not normal.

Levine et al. ('33) obtained weekly gains of 10 to 12 gm. during 5-week experiments in which a modified Steenbock rachitic ration was fed. This ration contained 14–17 parts of iodine per billion and furnished an average of 0.15 μg. per rat per day. These workers were able to prevent goiter in rats on this ration by adding iodine as potassium iodide to furnish a daily supply of 1 to 2 μg. per rat.

Remington ('37) incorporated dried pig liver into a modification of the Steenbock rachitic diet. Young rats grew 20 gm. per week on this ration. Rats reared on this ration grew to maturity and produced normal numbers of living young. In spite of the fact that the 2.0% liver contributed 0.04 μg. of iodine per day per rat, the thyroid enlargement was 3.7 times the normal as compared to 2.4 times the normal when no liver was included in the ration. It was found that 2.0% dried brewer's yeast gave the same growth increase as 1.0% dried pig liver in the modified Steenbock rachitic ration. The addition of yeast, which contained an insignificant amount of iodine, resulted in increased growth without affecting the degree of thyroid enlargement; hence, the increased thyroid hyperplasia caused by various levels of pig liver cannot be attributed to the increased rate of growth. Remington explains the goitrogenic effect of liver as being caused by the presence in liver of a substance the metabolism of which causes a drain on the thyroid mechanisms or of a specific thyroid stimulating hormone. Remington and Remington ('38) were able to reduce the hyperplastic thyroids of rats reared on the 2.0% pig liver ration to near normal by feeding 5–6 μg. of iodine as sodium iodide daily.

The type of ration consumed, apparently, has a marked effect on the rat's iodine requirement. In the present experiment, the thyroids of the rats receiving the 1.0 and 2.0 μg. supplements had an average weight of 10.2 mg. per 100 gm. of rat weight as compared to 8.8 ± 0.4 mg. per 100 gm. which, according to Remington, is the normal thyroid weight for 185-gm. rats.

Histological studies showed that 1 and 2 μg. supplements did not entirely correct the thyroid pathology. The pathological changes observed in the thyroid glands of the rats which received the basal ration only were much more extensive than has been previously reported by Hellwig ('32, '35), Thompson ('32), and Remington and Remington ('38). The possibility that soybean globulin contains a substance which causes an excessive strain on the thyroid mechanism either through increased metabolic demands or through direct stimulation cannot be ignored. However such a substance can hardly be the goitrogenic factor reported by Wilgus et al. ('41) which they found to be destroyed by heat, because the soybean protein we used was autoclaved. With respect to the Sharpless ('38) report that this goitrogenic substance can be extracted with fat solvents, a finding that Wilgus and associates were unable to confirm, it should be pointed out that the soybean protein we used was free from fat.

SUMMARY

A ration is reported which will produce an iodine deficiency in the white rat. Definite enlargement, redness, and histological changes were noted in the thyroid glands of the rats on the basal ration and on the basal plus 0.5 μg. of iodine per rat per day. The addition of 1.0 and 2.0 μg. of iodine per day to the basal ration largely corrected the glandular enlargement. The pathological changes in the thyroids were partially prevented by 1.0 μg. of iodine per day; however, 2.0 μg. resulted in almost complete prevention of pathological changes. No differences in growth or food consumption were noted between the basal group and the groups on the iodine supplements.

LITERATURE CITED

BARNES, R. H., AND J. E. MAACK 1943 The Hormel Institute of the University of Minnesota, Review: Nutritive Value of Soybeans.

HELLWIG, C. A. 1932 Iodine deficiency and goiter. Influence of a diet poor in iodine on the thyroid gland in white rats. Arch. Path., vol. 11, p. 709.

——— 1935 Experimental goiter. Functional, chemical and histological studies. Arch. Path., vol. 19, p. 364.

KRAUS, W. E., AND C. MONROE 1930 A comparison of the influence of iodized milk and potassium iodide administered directly, on the size and iodine content of the thyroid gland of rats. J. Biol. Chem., vol. 89, p. 581.

LEVINE, H., R. E. REMINGTON AND H. VON KOLNITZ 1933 Studies on the relation of diet to goiter. I. A dietary technic for the study of goiter in the rat. II. The iodine requirement of the rat. J. Nutrition, vol. 6, p. 325.

REMINGTON, R. E. 1937 Improved growth in rats on iodine deficient diets. J. Nutrition, vol. 13, p. 223.

REMINGTON, R. E., AND J. W. REMINGTON 1938 The effect of enhanced iodine intake on growth and on the thyroid glands of normal and goitrous rats. J. Nutrition, vol. 15, p. 539.

SHARPLESS, GEORGE R. 1938 A new goiter-producing diet for the rat. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 166.

TERESI, J. D. 1943 The nutritional study of the minor elements molybdenum, boron and iodine. Ph. D. thesis, University of Wisconsin, p. 46.

THOMPSON, J. 1932 The effect of rachitogenic diets on the thyroid gland of the albino rat. J. Nutrition, vol. 5, p. 359.

WILGUS, H. S., F. X. GASSNER, A. R. PATTON AND R. G. GUSTAVSON 1941 The goitrogenicity of soybeans. J. Nutrition, vol. 22, p. 43.

PLATE 1

EXPLANATION OF FIGURES

HISTOLOGICAL DESCRIPTIONS

1 A section of the thyroid gland of a rat which received the basal ration only. Epithelial hypertrophy. H and E. × 440.

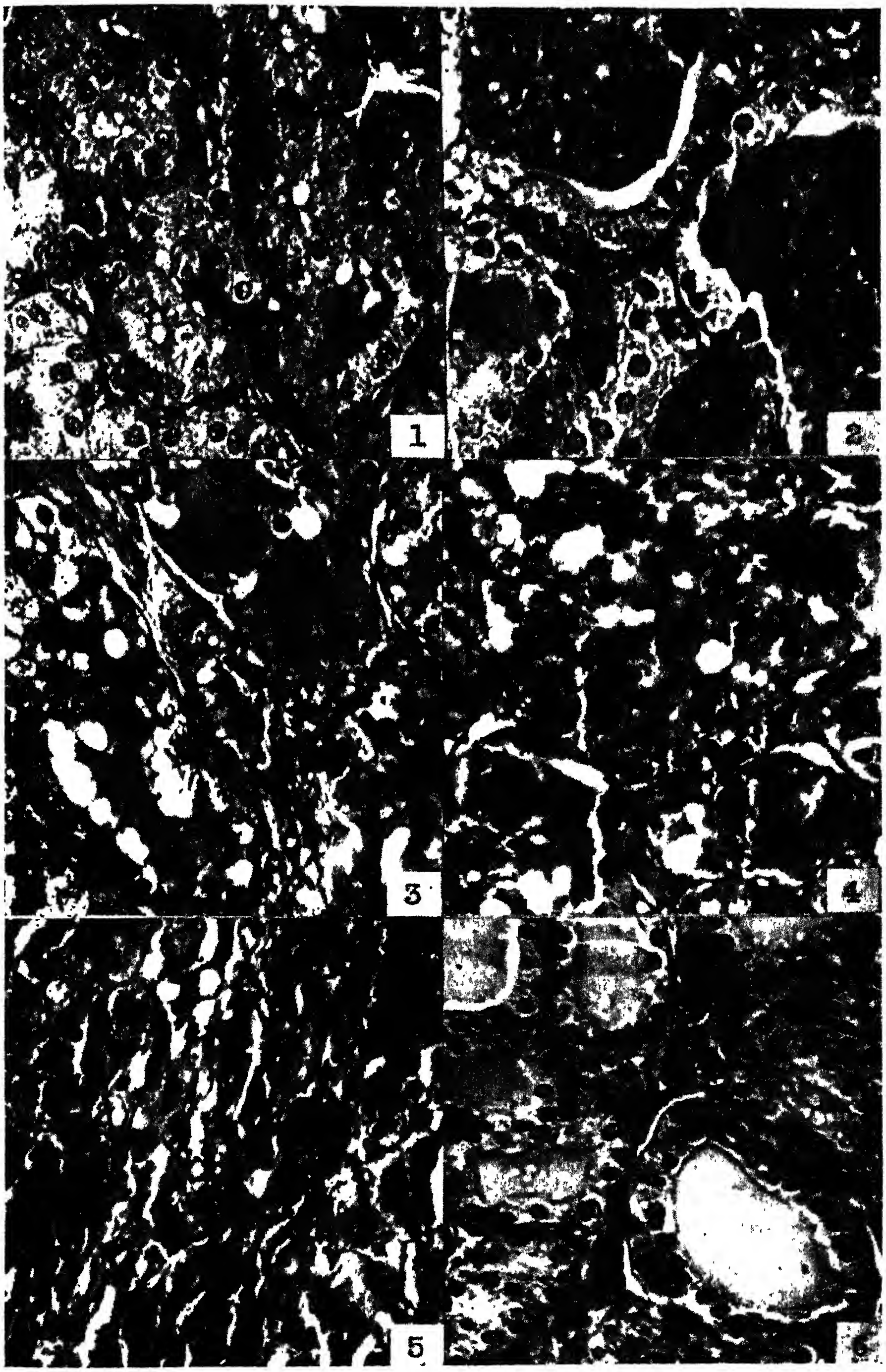
2 A section of the thyroid gland of a rat which received the basal ration plus 1.0 μg. of iodine per day. Epithelial hypertrophy and necrotic nuclei in the acinar lumina. H and E. × 440.

3 A section of the thyroid gland of a rat which received the basal ration only. Beginning of epithelial necrosis. H and E. × 440.

4 A section of the thyroid gland of a rat which received the basal ration only. More extensive epithelial necrosis. H and E. × 440.

5 A section of the thyroid gland of a rat which received the basal ration only. Advanced and widespread necrosis. H and E. × 440.

6 A section of the thyroid gland of a rat which received the basal ration plus 2.0 μg. of iodine per day. Normal. H and E. × 440.


1
2
3
4
5

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

I. THE HUMAN BIOASSAY TECHNIC [1]

DANIEL MELNICK, MELVIN HOCHBERG AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

ONE FIGURE

(Received for publication April 11, 1945)

Among the urgent needs for further research reported at the National Nutrition Conference in 1941 was "Improvement of presently known chemical and biological procedures for estimating the amounts of the essential nutrients in foods and their physiological availability." In this statement there are recognized not only the advantages of chemical and microbiological assays for vitamins, a field of methodology in which outstanding progress has been effected, but also the basic principle that content and availability are not synonymous terms in the case of vitamins any more than they are with respect to minerals, proteins, or carbohydrates. Analytical methods are directed toward establishing the total quantity of a specific nutrient present in a food. On the other hand, that fraction of the total nutrient which is available for absorption is measured by biological tests preferably on the animal species for which the food is intended. The need for further studies along these lines has recently been emphasized (Hart, '45).

Vitamins may exist in nature in such firm union with proteins or other compounds that they either fail to react chemically or are not utilized by the test microorganism until released by preliminary hydrolysis. This pretreatment of the test sample may not be duplicated by the digestive processes during the gastrointestinal journey.

Animal assays are usually regarded as measuring physiologically available nutrients, but they are not always reliable when applied to human nutrition. Among the factors affecting animal assays are the need for subdivision or extraction of the test materials, the pathologically depleted state of the experimental animal, the use of purified or

[1] This paper is one in a series of reports dealing with the physiological availability of the vitamins. It was presented in part before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of this study were defrayed by a grant from Lever Brothers Company, Cambridge, Massachusetts.

unbalanced basal rations whose composition may be specifically related to the utilization of the factor under test; these depart considerably from the conditions underlying the nutrition of man, and therefore warrant the exercise of caution in the application of laboratory data.

The present report describes the procedure for the assay of physiologically available vitamins, or for the study of conditions affecting their availability, directly on human subjects. The technic permits the simultaneous assay of the various water-soluble vitamins in foods or pharmaceutical products in the condition in which they are normally consumed. Since the test materials are fed in a single dose, it is possible to assay perishable materials without making assumptions as to stability such as are often necessary in prolonged animal assays. Evaluation of the rate as well as degree of availability can be made by this procedure. In cases where repetition of the control period may be dispensed with, the entire assay can be completed within 1 week. The precision of the human bioassay is at least as good as that of most animal assays.

Using normal subjects as the basis for comparison, it is possible by this technic to determine the effect of various conditioning factors (Jolliffe, '43), both physiological and pathological, on the availability of the water-soluble vitamins. Since the procedure in its present form is based on a standardized urinary response, its application is limited to those vitamins normally excreted via the renal route, namely, the water-soluble vitamins. A number of studies demonstrating the versatility and the reliability of the new assay technic have been completed and are being presented for publication.

EXPERIMENTAL PART

Principle of bioassay technic. In normal human subjects the urinary excretion of the water-soluble vitamins, as such or as their derivatives, is directly proportional to the quantity consumed, provided that at the time of the tests the subjects are subsisting on an adequate diet. The linear dose-response relationship established by feeding the vitamins in pure solution, i.e., in their most completely available form, constitutes the basis of the bioassay for the estimation of available ascorbic acid, thiamine, riboflavin and nicotinamide.

Collection of urine samples and analytical methods. The 24-hour urine samples are collected in opaque bottles containing 20 ml. of 3.5 N sulfuric acid and stored at room temperature. The ascorbic acid analyses are conducted immediately after completion of the samples. The

thiamine, riboflavin and N^1-methylnicotinamide in these samples are stable for a period of at least 2 weeks.

Ascorbic acid may be found in the urine as such and as the partially oxidized form, dehydroascorbic acid. The conversion of reduced to dehydroascorbic acid occurs not only in vivo (Berryman, French, Harper and Pollock, '44) but also during storage of the urine samples. It is markedly inhibited by low pH. The photometric procedure of Bessey ('38) as modified by Hochberg, Melnick and Oser ('43) has been used to determine both forms of urinary ascorbic acid. In this and subsequent reports only values for total ascorbic acid are reported. In general, about 90% of the total excreted vitamin is found in the reduced state, the remainder being dehydroascorbic acid.

Thiamine is excreted in the urine as the free vitamin (Melnick and Field, '39). It has been estimated by the simplified colorimetric method (Hochberg and Melnick, '44). Riboflavin has been determined by a modification (Strong and Carpenter, '42) of the microbiological method of Snell and Strong ('39) and by the fluorometric procedure described by Najjar ('41) omitting the blank correction for non-riboflavin fluorescent compounds. The microbiological procedure is more specific for riboflavin in urine than the abbreviated fluorometric procedure. However, the latter is far more precise and since conclusions are based upon the extra urinary excretion of the vitamin following riboflavin dosage, the calculations themselves correct for the presence of the irrelevant fluorescent materials in the two consecutive urine samples. It has also been found that the extra urinary excretions of riboflavin estimated by microbiological and fluorometric analyses give the same average values, but the figures for the individual test subjects vary more widely when the microbiological method is employed. N^1-Methylnicotinamide, the main excretory product following nicotinamide ingestion (Najjar and Wood, '40; Huff and Perlzweig, '43) has been determined fluorometrically (Hochberg, Melnick and Oser, '45).

Basal diet. The composition of the basal ration used in this and subsequent studies is shown in table 1. Analyses were conducted on aliquots of a composite sample of the diet. All foods containing active enzymes were first blanched under an atmosphere of nitrogen and the cooked foods, together with the original blanching fluids, were homogenized likewise under nitrogen. Aliquots were then promptly taken for the tests. Proximate analyses were conducted by the methods of the Association of Official Agricultural Chemists ('40). The same photometric procedure employed for the urine analyses was used for the determination of the ascorbic acid content of the diet. Riboflavin was

estimated by the microbiological procedure (Snell and Strong, '39); thiamine by the thiochrome method (Hennessy, '41); and nicotinic acid colorimetrically as described by Melnick ('42).

The content of the essential nutrients, listed in table 1, indicates that the basal diet furnishes sufficient protein of excellent quality and considerably greater quantities of the water-soluble vitamins than are

TABLE 1

Composition of basal diet.

	Breakfast		
1 orange	150 gm.	2 butter squares	14 gm.
2 slices of toast (enriched)	50 gm.	1 glass milk	230 gm.
	Dinner		
1 steak (lean)	150 gm.	5 butter squares	35 gm.
1 serving of fried potatoes	65 gm.	1 glass of milk	230 gm.
1 serving of carrots	60 gm.	1 serving of apple pie	155 gm.
1 serving of beets	70 gm.	2 hard sugar candies	12 gm.
2 slices of toast (enriched)	50 gm.		
	Supper		
2 fried eggs	90 gm.	1 glass of milk	230 gm.
1 lettuce serving	25 gm.	1 apple	150 gm.
1 tomato serving	70 gm.	1 banana	150 gm.
2 slices of bread (enriched)	66 gm.	2 hard sugar candies	12 gm.
4 butter squares	28 gm.		

Analyses conducted on aliquots of the composite diet.

Proximate analysis	*Values found*[1]	*Vitamin content*	*Values found*[1]
Total weight	2075 gm.	Thiamine	1.34 mg.
Total solids	540 gm.	Thiamine: Calorie ratio	0.5
Moisture	1535 gm.	Thiamine: Non-fat calorie ratio	0.9
Protein	101 gm.	Ascorbic acid	115 mg.
Fat (ether extract)	129 gm.	Riboflavin	2.54 mg.
Ash	22 gm.	Nicotinic acid[2]	22.0 mg.
Crude fiber	6 gm.		
Carbohydrate (by difference)	282 gm.		
Caloric value	2710 Cal.		
Non-fat calories	1550 Cal.		

[1] Expressed in terms of total food consumed in the 3 meals.

[2] Predominantly as the amide.

generally regarded as minimal for adequate nutrition. The thiamine: calorie ratio (Cowgill, '34) and the thiamine: non-fat calorie ratio (Williams and Spies, '38) are adequate for proper nutrition.

Selection of test subjects. For the availability studies it is desirable to use at least five subjects, preferably males. Inasmuch as deficient subjects retain extra dietary vitamin, a reliable ratio of extra urinary

excretion indicative of availability can be obtained only with subjects whose nutritional status is normal. Dietary histories obtained from daily records of food consumption kept by the prospective subjects yield first approximations on the suitability of the individuals for the study. However, such an approach is known to be of limited value, since dietary histories may very often be untrustworthy. For this reason it is essential to rely primarily on objective vitamin clearance tests for the selection of the subjects. Those employed for the present availability studies subsisted regularly on diets furnishing nutrients in amounts comparable to those listed in table 1 for the basal diet and excreted the vitamins well within the normal range both before and after postprandial dosage with extra vitamins. Typical urinary excretion values obtained with these subjects are given in table 2.

TABLE 2

Urinary excretion of vitamins (or derivatives) by test subjects.
(All values in milligrams.)

SUBJECT	SURFACE AREA IN SQ. M.	EXCRETION [1] OF VITAMIN OR DERIVATIVE, PER 24 HRS.							
		Thiamine		Ascorbic acid		Riboflavin		N^1-methylnicotinamide [3]	
		Basal	Increment after test dose [2]	Basal	Increment after test dose [2]	Basal	Increment after test dose [2]	Basal	Increment after test dose [2]
J.C.	1.70	0.33	1.03	18	121	0.73	4.26	8.0	14.7
E.M.	1.76	0.42	0.62	18	101	0.78	4.50	7.2	13.6
D.M.	1.88	0.39	0.93	36	117	0.57	4.84	7.7	9.8
M.H.	1.96	0.27	1.04	60	113	0.71	4.65	4.8	5.2
H.H.	2.15	0.31	0.84	52	121	0.74	4.70	3.8	7.7
Average	1.89	0.34	0.89	37	115	0.71	4.59	6.3	10.2

[1] On the day that urine samples were collected, each subject consumed the diet shown in table 1.

[2] The test dose of 5 mg. thiamine, 200 mg. ascorbic acid, 10 mg. riboflavin and 50 mg. nicotinamide in pure solution was taken orally immediately after dinner.

[3] Results expressed as nicotinamide on an equimolar basis.

Description and experimental justification of the assay technic. In biological assays with laboratory animals the same standardized diet is ingested daily throughout the period of observation. Such a technic cannot be applied readily to humans employed for bioassay purposes. For this reason marked simplification of the feeding routine is necessary. It has been found sufficient to have the test subjects consume the basal diet (see table 1) only on the days that urinary collections are made, allowing them to return to their customary but still adequate diets during the intervening periods. Under such circumstances monotony in the dietary is avoided and the routine feeding so simplified

that no difficulties are encountered in obtaining full cooperation from the test subjects.

Details of the method for conducting biological assays with human subjects can best be described by reference to a simple example. The problem was to determine the availability of the water-soluble vitamins in a pharmaceutical vitamin tablet when the product is swallowed whole.[2] Obviously disintegration in vivo is a necessary preliminary to absorption. The rat cannot be relied upon to measure availability since the intact tablet is too large to be administered. Bioassays with even larger laboratory animals would also be unsatisfactory since these would involve the feeding of curative or prophylactic doses of the vitamins in amounts far less than those contained in a whole tablet.

As an example of the data obtained in determining the availability of a vitamin, a test for ascorbic acid is summarized in table 3. Five

TABLE 3

Availability to man of ascorbic acid in a multivitamin-mineral tablet.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 200 MG. ASCORBIC ACID			TEST PERIOD: 15 TABLET DOSE 173 MG. ASCORBIC ACID		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	57	183	63	35	116	47
E.M.	41	159	59	18	92	43
D.M.	47	131	42	38	128	52
M.H.	70	162	46	17	96	46
H,H.	28	93	33	20	109	52
Average	49	146	48.6	26	108	48.0

Availability of ascorbic acid in tablets $= \frac{48.0}{48.6} \times 100 = 98.8 \pm 11.9\%$.

test subjects were employed for the assay. The basal urinary excretions of the vitamin on the planned adequate diet (table 1) were measured. Immediately after the mid-day meal, the subjects were given an aqueous solution of ascorbic acid[3] and the extra urinary excretion of

[2] The War Food Administration includes in certain contract specifications the proviso that the vitamins must be present not only in amounts to meet label claims, but that these factors must also be available when tablets or capsules are swallowed intact (Federal Surplus Commodities Corporation, '44).

[3] Actually the pure solution dose of ascorbic acid also contained the other water-soluble vitamins for which assays were being made. Studies have indicated that the extra urinary excretion of the water-soluble vitamins (or derivatives), thiamine, ascorbic acid, riboflavin and N^1-methylnicotinamide, by the normal subject subsisting on the basal diet are the same regardless of whether the vitamin is taken alone or in various combinations. It is important that the test doses of extra vitamins be taken postprandially, as indicated, or non-reproducible excretion values may be obtained; variations in the stability of some vitamins and in the rates and degrees of absorption of others are noted at different stages of gastrointestinal function.

the vitamin measured. The proportion of the test dose found in the 24-hour urine sample was then calculated; in this case it was 49% of the 200 mg. extra ascorbic acid taken. A 2 weeks' period was allowed to elapse during which the subjects returned to their original state of nutrition. The procedure was then repeated, but in place of the pure solution of the vitamin, the test material, containing a comparable quantity of ascorbic acid, was taken.[4] In this example, fifteen tablets were swallowed whole. The extra urinary excretion was then measured and the per cent of the test dose found in the urine was calculated. In the assay described extra quantities of ascorbic acid following tablet dosage could appear in the urine only after extraction of the vitamin from the disintegrated tablet and absorption from the gastrointestinal tract. Since the mean percentage of the test dose excreted after ingestion of the tablets equaled that when the vitamin was administered in the control solution, it was concluded that the ascorbic acid in the vitamin tablets was completely available.

The individual data for the test subjects in the ascorbic acid assay were actually not as reproducible from control to test period as the data for the other water-soluble vitamins, particularly with respect to the basal excretion values. However, it was precisely this greater variability in the ascorbic acid assay which led us to select it for detailed presentation because it demonstrates that such variations in the basal excretion values introduce no error in the assay. Nevertheless it is worthy of note that even in this example the standard error of the ratio of excretion in the two periods was only about 12% which is less than the error of most animal assays.

At first glance the discrepancies in the urinary excretion responses appear to be large. For example, M. H. excreted a total of 162 mg. of ascorbic acid during the control period but only 96 mg. during the test period. However, the basal value for the subject during the control period was markedly greater than that during the subsequent test period so that the same percentages of the test dose were excreted in the urines. Inasmuch as the basal figure may show this large variation from one period to another, the question may be raised — What justification is there for subtracting the basal urinary excretion from the total value to arrive at an amount attributable to the extra dosage? Data illustrating the reproducibility of the basal excretion on consecu-

[4] If the object is to study some conditioning factor which might interfere with the absorption of the vitamin, the extra vitamins are also taken during the test period in pure solution but with the imposition of the conditioning factor. Other modifications in the assay technic are frequently necessary as illustrated in forthcoming publications.

tive days are shown in table 4. Daily basal excretion values were obtained for the five subjects during different periods of 2 and 3 days, respectively. It will be noted that M. H. excreted far less ascorbic acid during period A than during period B but that the basal figures are reproducible within each period. Similar results were obtained with all of the subjects with respect to basal excretion values not only

TABLE 4

Reproducibility of basal urinary excretion values on consecutive days.
(All values in milligrams per 24 hours.)

FACTOR EXCRETED	SUBJECT	PERIOD A			PERIOD B			
		1st day	2nd day	Average	1st day	2nd day	3rd day	Average
Ascorbic acid	J.C.	36	45	41	36	45	57	46
	E.M.	17	12	15	74	61	59	65
	D.M.	56	38	47	40	44	43	42
	M.H.	18	17	18	67	59	57	61
	H.H.	29	20	25	22	23	27	24
	Average	31	26	29	48	46	49	48
Thiamine	J.C.	0.35	0.33	0.34	0.22	0.23	0.23	0.23
	E.M.	0.45	0.42	0.44	0.25	0.19	0.19	0.21
	D.M.	0.37	0.39	0.38	0.25	0.29	0.28	0.27
	M.H.	0.30	0.27	0.29	0.19	0.24	0.22	0.22
	H.H.	0.29	0.31	0.30	0.32	0.28	0.26	0.29
	Average	0.35	0.34	0.35	0.25	0.25	0.24	0.25
Riboflavin	J.C.	0.64	0.74	0.69				
	E.M.	0.85	0.95	0.90				
	D.M.	0.65	0.69	0.67				
	M.H.	0.75	0.84	0.80				
	H.H.	0.63	0.68	0.66				
	Average	0.71	0.78	0.74				
N^1-Methylnicotinamide (as nicotinamide)	J.C.	7.8	6.2	7.0				
	E.M.	6.7	5.9	6.3				
	D.M.	6.7	6.7	6.7				
	M.H.	3.8	4.5	4.2				
	H.H.	3.7	3.0	3.4				
	Average	5.8	5.2	5.5				

for ascorbic acid but for thiamine, riboflavin and N^1-methylnicotinamide as well. In any event the small daily variations within a given period are inappreciable in terms of the extra excretions following dosage. These findings justify the subtraction of the basal value on 1 day from the total excretion on the subsequent day to arrive at the quantity of extra urinary vitamin (or derivative) directly related to dosage.

In order to employ the ratio of the extra urinary excretion of the vitamin during the test period to that during the control period as an index of availability, it is necessary to demonstrate that a reproducible linear relationship exists between dosage and response. Figure 1 illustrates that, with increasing intake of any one of the four water-soluble vitamins studied, there is an increase in its extra urinary excretion. Good agreement is obtained among the test subjects receiving the three test doses of ascorbic acid; only one value for one subject

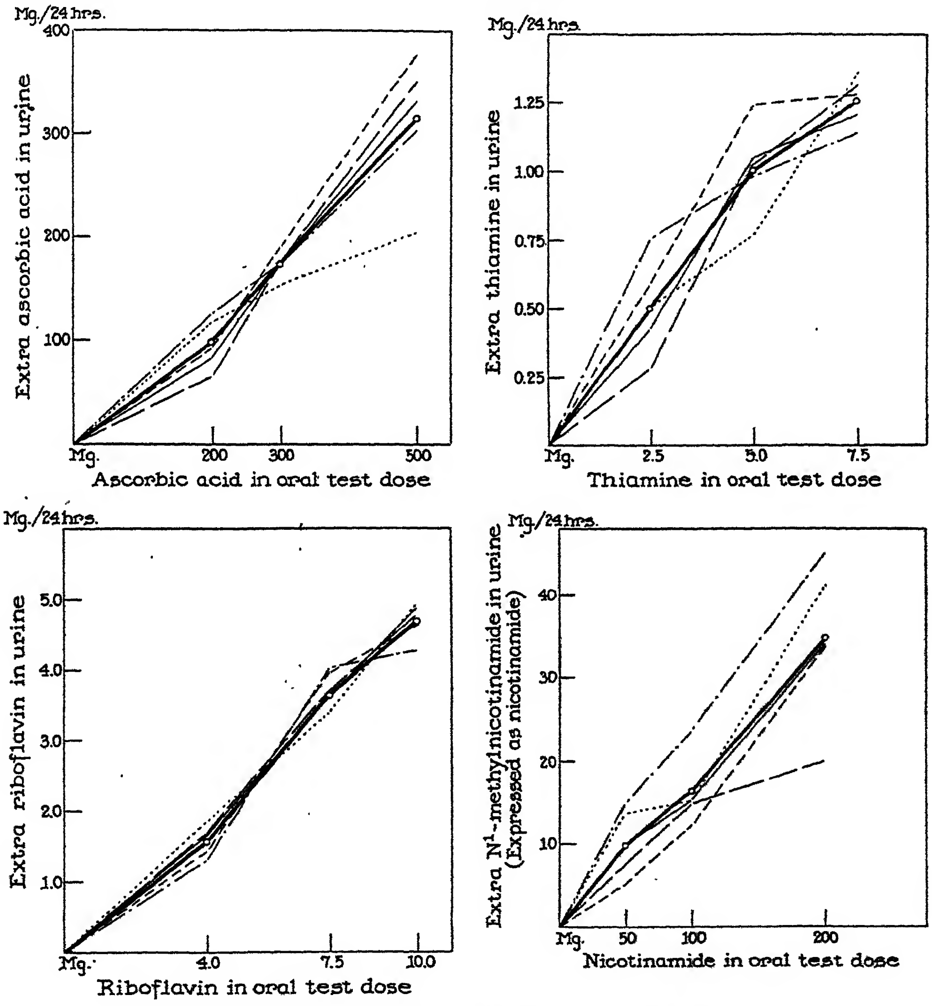


Fig. 1 The linear relationship between dosage with the water-soluble vitamins, ascorbic acid, thiamine, riboflavin and nicotinamide, and the extra urinary excretion of the vitamins (or derivative). The test doses in aqueous solution (pH 3.0) were taken orally immediately after dinner. The fine lines represent the responses of the individual subjects whereas the heavy line indicates the average response.

showed an appreciable deviation from all the others. In the case of thiamine the uniformity of linear response among the various subjects was not quite as good. However, in every case with increasing dosage there was an increment in the urinary output of the vitamin so that the average curve is linear. The dose-response relationship in the case of riboflavin is excellent, the values obtained with the five subjects being in very close agreement. The graph for the extra urinary excretion of N^1-methylnicotinamide following nicotinamide dosage is of interest. Although there was considerable spread among the responses of the test subjects, each individual was quite consistent. Thus, a subject who excreted appreciably more N^1-methylnicotinamide than another showed a greater urinary excretion at different levels of intake.

TABLE 5

Reproducibitily of the average urinary excretion responses following oral post prandial dosage with water-soluble vitamins.[1]

(All values in milligrams.)

VITAMIN TAKEN	DOSE	AVERAGE EXTRA URINARY EXCRETION OF VITAMINS AT DIFFERENT PERIODS			
Ascorbic acid	200	97	94	108	93
	300	173	150		
Thiamine	5.0	1.02	0.90	1.09	0.92
	7.5	1.26	1.26		
Riboflavin	7.5	3.8	3.6		
	10.0	4.7	4.9	5.6	5.3
Nicotinamide[2]	50	10.2	8.5	9.4	

[1] Each value listed represents the average of the responses by the five test subjects during the 24-hour period following dosage. These values were obtained over a period of 2 years.

[2] The excretion values following nicotinamide dosage represent the N^1methylnicotinamide found in the urine expressed as nicotinamide.

These dose-response curves were plotted from single urinary excretion values for each subject at the various levels of dosage. The mean curves for each subject derived from cumulative data are of course more nearly linear. However, in conducting an assay with a group of subjects the average urinary excretions in the single preceding control period are employed in calculating availability, as illustrated in the assay for ascorbic acid (see table 4). That these control data are sufficiently reproducible is shown in table 5, giving the average responses for the same five subjects taking different quantities of the vitamins at widely spaced periods. Only during the urinary collection periods

were the subjects fed the basal diet (see table 1); at other times they subsisted on their regular adequate dietaries.

DISCUSSION

Measurement of the urinary excretion of the water-soluble vitamins as a means for the early diagnosis of avitaminosis has been repeatedly criticized on the basis that it reflects only the immediate vitamin intake. This, however, is true only in the case of normal subjects since deficient individuals retain extra dietary vitamin to replenish depleted stores. This prompt response of normal subjects to variations in vitamin intake is utilized in the procedure here described for determining physiological availability. Since the water-soluble vitamins are not excreted to a significant extent in the feces, when administered as pure solutions in the doses selected, the present method does not involve analysis of stools. Thus, it is possible to avoid complications introduced by bacterial synthesis in the colon.

The assay technic described in this report is a simplification and standardization of procedures previously employed. Using the values for the urinary excretion of thiamine as indices of availability, Melnick, Robinson and Field ('41) were able to demonstrate that the increased requirements of peptic ulcer patients were not due to a decrease in availability of the vitamin because of the high fat content of the Sippy diet, but to the effects of the accompanying antacid medication. Subsequently Parsons and associates ('42 a, '42 b, '44) demonstrated also by urinary excretion studies that the vitamins of the B complex in fresh yeast were available only to a limited extent, and more recently (Williamson and Parsons, '45) that an increase in the crude fiber content of the ration did not reduce thiamine availability. In those as well as in our own studies, each subject served as his own control.

In studies with various pharmaceutical products (Oser, Melnick and Hochberg, '45) it has been found that the technic permits simultaneous assays for several vitamins and tests to be conducted on the materials in the physical state in which they are taken by the human, i.e., without requiring prior extraction or subdivision. By determining the urinary excretion values for consecutive 4-hour periods following dosage, the rate as well as degree of vitamin availability can be determined. In assays of standardized products it has been found that the values following pure solution dosage are so reproducible that repetition of the control series each time is unnecessary. Assays may then be completed within a week.

Further evidence for the reliability of the simplified assay technic described in this report has accumulated in studies, now in press, which involved application of the procedure to problems in human nutrition. These include investigations on the influence of the ingestion of live yeast, enzymes, certain ions, or adsorbents on the availability of the vitamins.

Because of their instability certain products, when subjected to rat assay, must be stored under conditions which prevent spoilage or be replaced by fresh samples at frequent intervals in the course of the assay. The present assay procedure, however, is of such short duration that no assumptions need be made as to the stability or reproducibility of the material from batch to batch.

SUMMARY

A biological assay technic has been described for the simultaneous determination of the physiological availability of the water-soluble vitamins, ascorbic acid, thiamine, riboflavin and nicotinamide. The test is based upon the observation that the urinary excretion of the water-soluble vitamins, as such or as their derivatives, parallels the quantity consumed provided that normal test subjects are employed and that the subjects at the time of the tests are subsisting on an adequate diet. A simplified feeding routine is described which extends the applicability of the assay to quantitative studies of problems in human nutrition, particularly those dealing with the factors which contribute to conditioned malnutrition. The dose-response relationships for each of the test vitamins have been found to be linear and the reproducibility of the data superior to that encountered in most animal assays.

LITERATURE CITED

Association of Official Agricultural Chemists 1940 Official and tentative methods of analysis of the Association.

Berryman, G. H., C. E. French, H. A. Harper and H. Pollack 1944 Response to the intravenous injection of ascorbic acid as indicated by the urinary excretion of the total and reduced forms. J. Nutrition, vol. 27, p. 309.

Bessey, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., vol. 126, p. 771.

Cowgill, G. R. 1934 Vitamin B requirement of man. Yale University Press, New Haven.

Federal Surplus Commodities Corporation 1944 Form SCP-1738, Program-GCP.

Hart, E. B. 1945 The chemist and the field of nutrition. Nutrition Rev., vol. 3, p. 33.

Hennessy, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal. Ed., vol. 13, p. 216.

Hochberg, M., and D. Melnick 1944 A simple modification of the colorimetric method for routine thiamine clearance tests. J. Biol. Chem., vol. 156, p. 53.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1943 Photometric determination of reduced and total ascorbic acid. Ind. Eng. Chem., Anal. Ed., vol. 15, p. 182.

——— 1945 Chemical determination and urinary excretion of the metabolite N^1-methylnicotinamide. J. Biol. Chem., vol. 158, p. 265.

HUFF, J. W., AND W. A. PERLZWEIG 1943 N^1-methylnicotinamide, a metabolite of nicotinic acid in the urine. J. Biol. Chem., vol. 150, p. 395.

JOLLIFFE, N. 1943 Conditioned malnutrition. J. Am. Med. Assn., vol. 122, p. 299.

MELNICK, D. 1942 Collaborative study of the applicability of microbiological and chemical methods to the determination of niacin (nicotinic acid) in cereal products. Cereal Chem., vol. 19, p. 553.

MELNICK, D., AND H. FIELD, JR. 1939 Chemical determination, stability and form of thiamine in urine. J. Biol. Chem., vol. 130, p. 97.

MELNICK, D., W. D. ROBINSON AND H. FIELD, JR. 1941 Fate of thiamine in the digestive secretions. J. Biol. Chem., vol. 138, p. 49.

NAJJAR, V. A. 1941 The fluorometric determination of riboflavin in urine and other biological materials. J. Biol. Chem., vol. 141, p. 355.

NAJJAR, V. A., AND R. W. WOOD 1940 Presence of a hitherto unrecognized nicotinic acid derivative in human urine. Proc. Soc. Exp. Biol. Med., vol. 44, p. 386.

OSER, B. L., D. MELNICK AND M. HOCHBERG 1945 Physiological availability of the vitamins. VII. Comparison of various technics for determining vitamin availability in pharmaceutical products. Ind. Eng. Chem., Anal. Ed., in press.

PARSONS, H. T., AND J. COLLORD 1942 a Human utilization of thiamin and riboflavin in yeast. J. Am. Diet. Assn., vol. 18, p. 805.

PARSONS, H. T., J. COLLORD, J. GARDNER, F. M. STRONG AND W. H. PETERSON 1942 b Utilization by man of the vitamin B complex in fresh yeast. Federation Proc., vol. 1, p. 129.

PARSONS, H. T., A. FOESTE, A. WILLIAMSON AND H. STETTLER 1944 The utilization of thiamine from four yeasts. Federation Proc., vol. 3, p. 96.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., vol. 11, p. 346.

STRONG, F. M., AND L. E. CARPENTER 1942 Preparation of samples for microbiological determination of riboflavin. Ind. Eng. Chem., Anal. Ed., vol. 14, p. 909.

WILLIAMS, R. R., AND T. D. SPIES 1938 Vitamin B_1 (thiamin) and its use in medicine. Macmillan Co., New York.

WILLIAMSON, A., AND H. T. PARSONS 1945 Some factors influencing the fecal elimination of thiamine by human subjects. J. Nutrition, vol. 29, p. 51.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

II. THE EFFECT OF DIETARY THIAMINASE IN FISH PRODUCTS [1]

DANIEL MELNICK, MELVIN HOCHBERG AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Chastek paralysis, an acute dietary disease of foxes, is caused by including 10% or more of certain species of uncooked fish in the diet and may be prevented or cured by giving adequate amounts of thiamine (Green and associates, '42). The enzymic nature of the anti-thiamine factor is indicated by its heat-lability (Sealock and collaborators, '43), by its reactions in the presence of enzyme inhibitors (Sealock and Goodland, '44), and by the isolation of the pyrimidine and thiazole derivatives following the hydrolytic cleavage of the vitamin (Krampitz and Woolley, '44).

On the basis of the study conducted by Deutsch and Hasler ('43) it has been generally concluded that the anti-thiamine factor is found only in fresh-water fish. These investigators reported the presence of this factor in fifteen out of thirty-one species of fresh-water fish but in not one of nine species of salt-water fish. Others, however, have found it in the Atlantic herring and whiting and in the Pacific mackerel. In addition, thiamine deficiency has been observed in cats fed a diet consisting exclusively of salt-water herring (Smith and Proutt, '44).

In discussing the subject of thiamine inactivation by raw fish, Nutrition Reviews ('43) states, "This subject may not appear to have a direct bearing on human nutrition since it is unlikely that many people are fond of eating uncooked fish." However, there are a number of popular fish products which do not undergo excessive heat processing and are intended for direct human consumption. Woolley ('43) has reported the presence of a substance in clams capable of destroying thiamine. Clams are consumed in the raw state as are also oysters, herring, and caviar. Smoked fish may be processed at as low temperatures as 27° to 65°C.

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

In the present study an investigation was made of the presence of the thiaminase in various fish (including shell-fish) products and the influence of this factor on the physiological availability of dietary and supplementary thiamine.

EXPERIMENTAL PART

In table 1 are presented the results of tests to determine the thiaminase activity of a variety of fish products intended for direct human consumption. Ten-gram samples of each product were suspended in 40 ml. of water and the mixture homogenized. One hundred micrograms of thiamine were then added and the suspension incubated for 6 hours at 37°C. The control series consisted of suspensions heated [2] to inactivate the enzymes.

TABLE 1

Thiaminase activity of fish products intended for direct human consumption.

PRODUCT	pH [1]	THIAMINE RECOVERED FROM SUSPENSION	
		Untreated material [2]	Heated material [3]
		%	%
Clams			
Chowder	7.0	3	89
Steamer	6.9	1	92
Cherrystone	7.1	0	95
Herring			
Salted	6.3	40	89
Marinated (brand A)	4.6	48	96
Marinated (brand B)	4.5	87	103
Oysters	6.6	102	96
Smoked carp	6.7	96	99
Smoked salmon	6.2	98	101
Salmon caviar	6.0	100	100

[1] Of homogenized aqueous suspension.

[2] 10-gm. samples of each ground product were suspended at their natural pH in 40 ml. of water containing 100 µg. of added thiamine, and incubated 6 hrs. at 37°C.

[3] 10 gm. of each of the homogenized samples were heated for 20 minutes in 10 ml. of boiling water at pH 4.5 to inactivate enzymes. To the cooled suspension, diluted to 40 ml. and adjusted to the initial pH, were added 100 µg. of thiamine.

The results, presented in table 1, indicate that homogenized clams are very effective in destroying thiamine and that the agent responsible

[2] Each suspension was heated for a period of 20 minutes at 100°C. and pH 4.5, then adjusted to its initial pH value and finally cooled to 37°C. Destruction of thiamine in the suspension of the untreated fish product over and above that occurring in the corresponding heated sample may be attributed to the presence of the thiaminase.

for the destruction of added vitamin is heat labile. Complete destruction of thiamine was obtained with all three varieties of clam tested, namely, chowder, cherrystone and steamer. Herring, a salt-water fish, also contained the enzyme but probably in smaller concentration. Only 60% destruction of the added thiamine was noted in a case of the raw product and variable losses with the marinated preparations. No evidence was found for the presence of thiaminase in oysters, salmon caviar, smoked carp and smoked salmon; theoretical recoveries of the added thiamine were obtained in the suspensions of untreated homogenized samples. Apparently smoke-processing of the carp destroys the thiaminase originally present in the tissues of this species.

On the basis of the results obtained with the various fish products, it was decided to limit the human availability study to an investigation of the effectiveness of the thiaminase derived from chowder clams. Additional in vitro experiments have also been conducted. The test subjects were the same as those employed in the previous study (Melnick, Hochberg and Oser, '45). The only major deviation in the composition of the basal ration from that routinely employed was inclusion of clams at each meal. The total quantity of clams consumed during the day, 100 gm., is usually regarded as an "average serving". The purpose in partitioning this serving among the three meals of the day was to allow a more measurable destruction of the thiamine in vivo, if such should be the case, since the clams would always accompany the dietary thiamine. Thiamine intake was determined by the thiochrome method (Hennessy, '41) and urinary excretion by the colorimetric procedures [3] (Melnick and Field, '39; Hochberg and Melnick, '44). In the earlier report (cited above) the rationale and experimental support for the human availability technic were discussed.

In vitro tests. In table 2 are presented the results of in vitro experiments on the activity of the thiaminase derived from clams. Two series of tests were conducted, both on the homogenized basal diet. For one series, the homogenized clams were first heated [4] in order to inactivate all enzymes present. For the other, the unheated ground clams were added to the homogenized ration. The dietary mixtures in open beakers

[3] The simpler colorimetric method (Hochberg and Melnick, '44) is preferred for measurements of the urinary thiamine when appreciable amounts are present. However, because conclusions were to be drawn in this study not only from the extra urinary thiamine following test dosage but also from the lower basal excretion values, the original colorimetric procedure (Melnick and Field, '39) was also employed.

[4] One hundred grams of the ground fresh clams were heated for 20 minutes in 100 ml. of boiling water at pH 4.5 to inactivate the thiaminase. The mixture was then adjusted to its initial pH of 7.0 and added to the homogenized diet.

were then incubated for 6 hours at 37°C. at their natural pH of 5.5 and gently stirred every half hour.

The results summarized in table 2 indicate that during the incubation of a homogenized ration containing the heated clam mixture, a loss of only 15% of the thiamine occurred. A comparable percentage loss was noted in the case of the added thiamine.

The addition of the raw clam suspension to the dietary mixture resulted in marked destruction of the thiamine. This occurred during the first 15 to 20 minutes required for the preparation of a uniform mixture for sampling. Subsequent incubation of this dietary mixture failed to reduce any further the value for residual thiamine. The addition of 7.5 mg. of thiamine to the homogenized basal diet containing the raw

TABLE 2

In vitro tests of the activity of thiaminase in clams.

EXPERIMENT		TOTAL THIAMINE FOUND IN DIET
No.	Conditions[1]	
		mg.
I	Homogenized basal diet containing heated[2] clams	1.34
II	1 incubated 6 hrs. at 37°C.	1.14
III	1 + 7.5 mg. of thiamine	8.30
IV	III incubated 6 hrs. at 37°C.	7.60
V	Homogenized basal diet containing raw clams	0.21
VI	V incubated 6 hrs. at 37°C.	0.21
VII	V + 7.5 mg. of thiamine	0.20
VIII	VII incubated 6 hrs. at 37°C.	0.20

[1] All homogenized mixtures were incubated at pH 5.5.

[2] 100 gm. of ground clams (flesh) were heated for 20 minutes in 100 ml. of boiling water at pH 4.5. The mixture was adjusted to its initial pH of 7.0 and then added to the homogenized diet.

clams resulted in rapid and complete destruction of the added vitamin. Incubation of this dietary mixture likewise failed to lower the residual thiamine content. It may very well be that of the total thiamine figure for the basal dietary mixture 0.2 mg., or 15%, represents non-specific reacting substances indistinguishable from thiamine in the thiochrome assay procedure. These experiments demonstrate that within 20 minutes one serving of clams (100 gm.) can destroy more than 8 mg. of thiamine, i.e., more than eight times the minimal daily requirment. However, factors capable of destroying ingested enzymes or of rendering them inactive are known to be present in the gastrointestinal tract. The effect of the ingestion of raw clams on the availability of thiamine, when disintegration of cells and liberation of the thiaminase occurs by virtue of

the digestive enzymes in the gastrointestinal tract, could only be determined by direct studies on man.

In vivo studies. The results of the study on human test subjects are presented in table 3. The control data were obtained with the individuals on the basal ration containing the heat-inactivated clam suspension.

During the first 3 days no extra thiamine was taken. On the fourth day a test dose of 7.5 mg. of the vitamin was administered postprandially, and on the fifth day the basal ration alone was again ingested. The responses of the subjects were quite uniform; good reproducibility was

TABLE 3

Urinary excretion of dietary and extra thiamine by subjects subsisting on a diet containing raw and heated[1] clams.

(All values in milligrams.)

SUBJECT	DIET CONTAINING HEATED CLAMS					NORMAL BASAL EXCRETIONS[2]	DIET CONTAINING RAW CLAMS[3]				
	Basal excretions			After 7.5 mg. of thiamine			Basal excretions			After 7.5 mg. of thiamine	
	1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.
J.C.	0.22	0.23	0.23	1.59	0.54	0.23	0.12	0.11	0.10	0.88	0.16
E.M.	0.25	0.19	0.19	1.33	0.33	0.21	0.12	0.09	0.07	0.59	0.25
D.M.	0.25	0.29	0.28	1.80	0.47	0.27	0.17	0.11	0.11	0.74	0.25
M.H.	0.19	0.24	0.22	1.02	0.38	0.22	0.15	0.14	0.11	0.85	0.25
H.H.	0.32	0.28	0.26	1.79	0.36	0.29	0.14	0.09	0.09	0.95	0.18
Average	0.25	0.25	0.24	1.51	0.42	0.24	0.14	0.11	0.10	0.80	0.22

[1] An aqueous suspension of ground clams at pH 4.5 boiled to inactivate the thiaminase.

[2] The average excretion of the corresponding subject subsisting on the diet containing the heated clams.

[3] The raw clams were served whole.

observed from day to day in their basal thiamine excretion, as well as an increased output on the day following the test dose and small carryover (second 24 hour) values. Two weeks later the experiment was repeated, but this time the diet included the clams in their natural raw state.[5] In every case there was a prompt and continuous decrease in the basal urinary excretion values as the subjects continued on the diet containing the raw clams. The increments in the urinary excretion values following dosage with 7.5 mg. of thiamine were consistently smaller than those noted when the thiamine solution was taken along with the heated clams. This relationship was also apparent in the carryover values for the two periods.

[5] The clams were chewed two or three times, as is customarily done, leaving disintegration of the cells and liberation of the thiaminase to gastrointestinal enzymic processes.

Table 4 is an evaluation of the data of the preceding table in order to obtain a quantitative index of the destructive action of the thiaminase in raw clams during gastrointestinal digestion. The decrease in the basal thiamine excretion values would indicate that 50% or more of the dietary thiamine is destroyed. This urinary excretion picture is comparable to that obtained with normal subjects during the first 3 days following a 70% reduction in the normal thiamine intake (Melnick, Field and Robinson, '39).[6] When the extra urinary responses for the control and test period are compared on the basis of per cent of test dose excreted, 42% destruction of the added thiamine is found to have occurred.

TABLE 4

Destruction of dietary and extra thiamine by thiaminase in clams.

SUBJECT	DIET CONTAINING HEATED CLAMS: Average basal excretion	DIET CONTAINING HEATED CLAMS: Fraction of 7.5 mg. test dose excreted, 1st 24 hrs.	DIET CONTAINING HEATED CLAMS: Fraction of 7.5 mg. test dose excreted, 2nd 24 hrs.	DIET CONTAINING RAW CLAMS: Change in basal excretion[1], 1st 24 hrs.	DIET CONTAINING RAW CLAMS: Change in basal excretion[1], 2nd 24 24 hrs.	DIET CONTAINING RAW CLAMS: Change in basal excretion[1], 3rd 24 24 hrs.	DIET CONTAINING RAW CLAMS: Fraction of 7.5 mg. test dose excreted, 1st 24 hrs.	DIET CONTAINING RAW CLAMS: Fraction of 7.5 mg. test dose excreted, 2nd 24 hrs.
	mg./24 hrs.	%	%	%	%	%	%	%
J.C.	0.23	18.1	4.1	— 46	— 50	— 56	10.3	0.8
E.M.	0.21	14.8	1.5	— 44	— 56	— 66	6.9	2.3
D.M.	0.27	20.4	2.6	— 36	— 58	— 60	8.4	1.9
M.H.	0.22	10.8	2.2	— 30	— 37	— 48	9.8	1.8
H.H.	0.29	20.0	0.9	— 53	— 68	— 68	11.4	1.2
Average	0.24	16.8	2.3	— 42	— 55	— 60	9.4	1.6
Average total excretion of extra thiamine		19.1%		..	..	..	11.0%	
Destruction of thiamine taken as test dose	$\frac{19.1-11.0}{19.1} \times 100 = 42\%$.							

[1] Based on the average excretions of the corresponding subjects receiving the diet containing the heated clams.

DISCUSSION

The extent of thiamine destruction in vivo is not as great as that observed in the in vitro experiments. This is undoubtedly due to the fact that in the latter tests the clams were added to the dietary mixture in a finely homogenized state, allowing immediate and more complete contact between thiamine and thiaminase in the static system. In the study with the human subjects the thiaminase was liberated slowly as the clams were digested by enzymic processes. Thus, the opportunity existed for absorption of some dietary thiamine and certainly of a significant fraction of the test dose prior to the liberation of appreciable amounts of the thiaminase.

[6] Subject, D. M., participated in both studies.

The effectiveness of the thiaminase in clams in destroying thiamine and the presence of this factor in other fish products (such as the herring) ordinarily consumed in the raw state represent a type of conditioning factor potentially responsible for malnutrition (Jolliffe, '43). The present study is far from being a survey of all fish products intended for direct human consumption, although it might be worthy of extension especially in relation to populations in those regions where fish, raw or partially cooked, constitute a major element of the diet. In any case, these observations indicate that the presence of the anti-thiamine factor in raw fish is not merely of academic interest but worthy of practical consideration in human nutrition.

The anti-thiamine effect of the homogenized clam suspension should find applications in methods, biological and chemical, designed to measure thiamine. In the former type of assay, the basal diet must supply all factors except thiamine. Autoclaving or sulfite treatment (U. S. Pharmacopoeia, '42) of the ingredients which furnish the B complex to insure complete destruction of thiamine, often involves concomitant destruction of other factors. The materials may preferably be incubated with a homogenized clam suspension to effect specific destruction of the thiamine. The blank value in the thiochrome procedure for determining thiamine (Hennessy, '41; U. S. Pharmacopoeia, '44) is frequently unsatisfactory since fluorescent materials may be present in the alkalinized solution which are destroyed on the addition of ferricyanide (Najjar and Ketron, '44). The sulfite treatment of the test solution (Mason and Williams, '42) has been reported to destroy interfering substances as well as thiamine thereby yielding low blanks (Najjar and Ketron, '44). However, the short incubation of a test extract with a small amount of an aqueous homogenized clam suspension (which is always free from thiamine) should allow specific destruction of thiamine leaving the true blank correction.

SUMMARY

In vitro tests have indicated that various fish products intended for direct human consumption contain a thiaminase which destroys thiamine in a static test system. Rapid and complete destruction of the vitamin occurred in vitro during incubation of food mixtures containing added thiaminase, as present in raw clams. More than eight times the minimal daily requirement of thiamine was destroyed simply in preparing a homogeneous mash of a daily ration to which both clams and thiamine had been added.

The results of the human availability study indicate that an appreciable destruction of thiamine (about 50%) occurs in the gastrointestinal tract following the concomitant ingestion of raw clams. Because other fish products are also consumed in a raw or partially cooked state by man, this finding warrants consideration of the anti-thiamine principle in fish products as a possible conditioning factor in malnutrition.

The application of the thiaminase in clams to vitamin B_1 methodology, both biological and chemical, has been discussed.

LITERATURE CITED

DEUTSCH, H. F., AND A. D. HASLER 1943 Distribution of a vitamin B_1 destructive enzyme in fish. Proc. Soc. Exp. Biol. Med., vol. 53, p. 63.

GREEN, R. G., W. E. CARLSON AND C. A. EVANS 1942 The inactivation of vitamin B_1 in diets containing whole fish. J. Nutrition, vol. 23, p. 165.

HENNESSY, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal. Ed., vol. 13, p. 216.

HOCHBERG, M., AND D. MELNICK 1944 A simple modification of the colorimetric method for routine thiamine clearance tests. J. Biol. Chem., vol. 156, p. 53.

JOLLIFFE, N. 1943 Conditioned malnutrition. J. Am. Med. Assn., vol. 122, p. 299.

KRAMPITZ, L. O., AND D. W. WOOLLEY 1944 The manner of inactivation of thiamine by fish tissue. J. Biol. Chem., vol. 152, p. 9.

MASON, H. L., AND R. D. WILLIAMS 1942 Determination of thiamine in urine by the thiochrome method: estimation of the blank. J. Biol. Chem., vol. 146, p. 589.

MELNICK, D., AND H. FIELD, JR. 1939 Chemical determination, stability and form of thiamine in urine. J. Biol. Chem., vol. 130, p. 97.

MELNICK, D., H. FIELD, JR. AND W. D. ROBINSON 1939 A quantitative chemical study of the urinary excretion of thiamine by normal individuals. J. Nutrition, vol. 18, p. 593.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

NAJJAR, V. A., AND K. C. KETRON 1944 An improved thiochrome method for the determination of thiamine in urine. J. Biol. Chem., vol. 152, p. 579.

NUTRITION REVIEWS 1943 Thiamine inactivation by raw fish. Vol. 1, p. 403.

SEALOCK, R. R., A. H. LIVERMORE AND C. A. EVANS 1943 Thiamine inactivation by the fresh-fish or Chastek-paralysis factor. J. Am. Chem. Soc., vol. 65, p. 935.

SEALOCK, R. R., AND R. L. GOODLAND 1944 Thiamine inactivation by the Chastek-paralysis factor. Inhibition of thiamine inactivation. J. Am. Chem. Soc., vol. 66, p. 507.

SMITH, D. C., AND L. M. PROUTT 1944 Development of thiamine deficiency in the cat on a diet of raw fish. Proc. Soc. Exp. Biol. Med., vol. 56, p. 1.

U. S. PHARMACOPOEIA 1942 Preparation of purified liver extract. Twelfth Revision, p. 626.

——— 1944 Thiochrome assay for thiamine hydrochloride. Twelfth Revision, First Bound Supplement, p. 82.

WOOLLEY, D. W. 1943 Personal communication.

COMPARATIVE MERITS OF FASTING SPECIMENS, RANDOM SPECIMENS AND ORAL LOADING TESTS IN FIELD NUTRITIONAL SURVEYS [1]

R. E. JOHNSON, C. HENDERSON, P. F. ROBINSON AND F. C. CONSOLAZIO

The Fatigue Laboratory, Harvard University, Soldiers Field, Boston

ONE FIGURE

(Received for publication March 12, 1945)

The systematic carrying out of nutritional observations on soldiers in the field is frequently difficult for military reasons, and it is at times almost impossible to collect samples of blood and urine under conditions and at times that are customarily considered almost essential for accurate nutritional assessment. Of the usual types of specimen,[2] one can almost always obtain fasting urine and blood before breakfast and at other times of day; sometimes conduct short vitamin loading tests; and almost never collect specimens of urine for periods of 24 hours. Many practical problems of conducting surveys would be made simpler if one knew accurately the quantitative relations between specimens collected before breakfast, after loading tests, and at random during the day.

The present study was carried out on soldiers living and working in temperature environments while subsisting on field rations, and the data support four main conclusions concerning men under such conditions. First, loading tests with ascorbic acid, thiamine, riboflavin and

[1] This work was financed in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College. The data were obtained in U. S. Army field trials to which the senior author was assigned as expert consultant for the Research and Development Branch, Military Planning Division, Office of the Quartermaster General. Permission to publish these findings has been granted by the Bureau of Public Relations, U. S. War Department, but the assertions and opinions expressed are those of the authors, and do not necessarily reflect the official position of any governmental agency.

[2] Throughout this paper several conventions of nomenclature will be followed. "Fasting" and "postabsorptive" are used synonymously to mean samples collected between the time of emptying the bladder on arising but before breakfast. "Postprandial" and "postcibal" are used synonymously to mean samples collected at any time after breakfast but before bedtime. "Loaded" refers to specimens collected after the administration of test doses of vitamins.

nicotinamide could be repeated on the same subjects at intervals of 2 or 3 weeks without significantly affecting their response to subsequent loading tests. Second, the rate of excretion of vitamins in the morning before breakfast was a more reliable indication of previous intake of vitamins than was the vitamin load test. Third, specimens of blood drawn at any time of day were directly comparable in several important respects with specimens drawn before breakfast. Fourth, even when due allowance was made for rate of urinary flow or urinary specific gravity samples of urine collected at various times of day did not give results for chloride, ascorbic acid, thiamine, N^1-methylnicotinamide or riboflavin that were directly comparable with results from samples collected before breakfast.

METHODS

Samples of blood and urine were collected in bivouac areas and analyses were carried out on the spot in a field laboratory. Whole blood hemoglobin was estimated by the copper sulfate specific gravity method of Phillips, Van Slyke and colleagues ('43); serum protein by the same method; serum and urinary chloride according to Harvey ('10); serum and urinary ascorbic acid according to Farmer and Abt ('36); and urinary thiamine, N^1-methylnicotinamide and riboflavin by fluorometric methods (Johnson, Sargent, Robinson and Consolazio, '45).

Observations were made on two separate groups under different conditions of diet, environment and activity which will be differentiated below. The subjects were all in good general health as judged by their medical officers.

RESULTS

A. Effects of repeated load tests

These were studied in a company (96 men) from an infantry battalion that lived and worked for 8 weeks in the summer in a bivouac area situated in the Rocky Mountains at an altitude of about 9000 feet. The daily temperatures ranged from near freezing at dawn to about 90°F. in the heat of the day, with infrequent thunder showers interrupting the otherwise clear, sunny weather. Activities included regular infantry training, cross-country marches, road marches, and tactical problems, with 1 day of rest a week, the whole summer's program involving an estimated average daily work expenditure of about 4000 Cal.

The soldiers all subsisted exclusively on a combat ration providing the following estimated average daily amounts of vitamins: ascorbic

acid, 65 mg.; riboflavin, 1.5 mg.; thiamine, 2.0 mg.; and niacin, 17 mg. Vitamin load tests were administered to 50 of the subjects on the first day of maneuvers, again 3 weeks later and finally at the end of 5 weeks. A vitamin load test was administered to the remaining forty-six subjects only once, at the end of the 5 weeks. The two groups were large enough so that comparison between them could be made on the assumption that their average nutritional status was the same except for the difference in load tests.

On test days fasting specimens of urine were collected between 4:45 A.M. and 6:15 A.M. and each subject then drank 150 ml. of water containing 5 mg. thiamine hydrochloride, 5 mg. riboflavin, 50 mg. nicotinamide, and 500 mg. ascorbic acid. Their usual breakfast was allowed and the subjects collected all their urine for 4 hours after the test dose. We use this schedule for tolerance tests in the field because breakfast has to be allowed for the sake of morale and a good morning's

TABLE 1

Fasting urinary levels in men who had and had not been subjected previously to vitamin load tests.

	NO PREVIOUS LOAD TEST (46 SUBJECTS)		LOAD TESTS 5 AND 2 WEEKS PREVIOUSLY (50 SUBJECTS)	
	Mean	*Range*	*Mean*	*Range*
Ascorbic acid (mg./hr.)	0.6	0.4 to 1.8	0.8	0.4 to 1.8
Thiamine (μg./hr.)	11	6 to 24	12	6 to 42
Riboflavin (μg./hr.)	27	12 to 68	22	8 to 93
N^1-methylnicotinamide (mg./hr.)	0.25	0.10 to 0.50	0.30	0.10 to 0.58

performance; furthermore intravenous tests on a large scale are not feasible in the field; also longer collection periods are usually impossible because of military necessities. The reliability of the 4-hour oral test in the case of vitamin C has been completely demonstrated for groups of subjects whose body stores were known to be very low, average and high by virtue of 8 weeks' subsistence on diets lacking, normal or high in vitamin C (Johnson, Darling, Sargent and Robinson, '45); and its validity in the case of thiamine, riboflavin and nicotinamide is demonstrated in a forthcoming paper (Johnson, Contreras, Robinson and Consolazio, '45).

The results for fasting urine, converted to hourly excretions from those of the actual collection periods, are summarized in table 1. The averages for ascorbic acid, thiamine and N^1-methylnicotinamide were not significantly different between the two groups and the average for riboflavin was higher in the group that had never previously received a load test. The range tended to extend a little higher in the group

that had received two previous load tests, but the distribution curves were not significantly different, except that riboflavin values were somewhat higher in the group that had never received load tests.

The comparison of the load tests was similar to that of the fasting specimens collected before breakfast. The averages for the groups that had and had not received load tests were: for ascorbic acid — 54 and 64 mg. in 4 hours after the test dose; for thiamine — 405 and 400 µg. in 4 hours; for N¹-methylnicotinamide — 4.75 and 4.75 mg. in 4 hours; and for riboflavin — 1575 and 1600 µg. in 4 hours. Inspection of the distribution curves (fig. 1) shows a fairly wide spread in both groups, but curves that are very similar in shape.

From this set of observations, it may be concluded that in the case of men on similar diets of reasonably high vitamin content, assays of

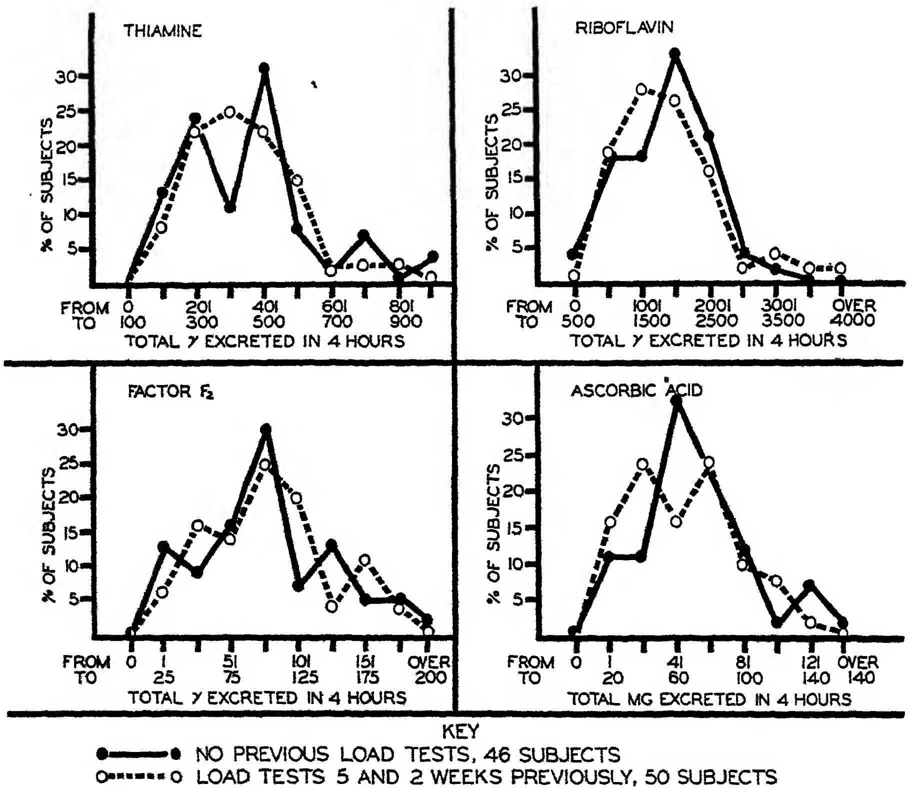


Fig. 1 Distribution curves for urinary excretion of thiamine, riboflavin, N¹-methylnicotinamide and ascorbic acid after oral test doses in subjects who had and had not previously received such tests. Ordinates are the percentage of subjects in categories represented by abscissae, which are the ranges of urinary excretion in 4 hours after test dose. (The factor for converting values for Factor F_2, which are expressed in micrograms, quinine units, into N¹-methylnicotinamide, milligrams, is $\frac{1}{20}$.)

the fasting urine before breakfast and of oral load tests of short duration are not significantly affected by load tests administered at intervals of 2 or 3 weeks. This conclusion should not be extended without further evidence to subjects on frankly deficient diets or to types of load test different from that used in this study.

B. Comparison of fasting urinary levels with results of load tests

This comparison was made between two platoons (26 and 31 men, respectively) of a company from the battalion described in Section A above. The diet was a field ration which provided the following estimated daily average intake of nutrients: total calories, 4100; protein, 140 gm.; ascorbic acid, 90 mg.; riboflavin, 2.5 mg.; thiamine, 2.1 mg.; and niacin, 27 mg. During the last 3 weeks of the test, platoon 1 received placeboes and platoon 2 vitamin pills providing daily: vitamin A, 7500 U.S.P. units; vitamin D, 600 U.S.P. units; ascorbic acid, 112.5 mg.; thiamine hydrochloride, 3.0 mg.; riboflavin, 4.5 mg.; and nicotinamide, 30.0 mg.

Under the present experimental circumstances the fasting specimen was a far more sensitive indication that the subjects had been receiving supplementary ascorbic acid, riboflavin, thiamine, and nicotinamide than was the oral load test (table 2). Highly significant differences be-

TABLE 2

Comparison between platoon 1, which received placeboes for 3 weeks, and platoon 2, which received vitamin pills.

	MEANS FOR PLATOON 1 (26 MEN)		MEANS FOR PLATOON 2 (31 MEN)	
	Before	Placeboes for 3 weeks	Before	Vitamin pills for 3 weeks
A. Urinary excretion in fasting state [1]				
Ascorbic acid (mg./hr.)	0.8	0.8	0.8	1.4
Thiamine (μg./hr.)	17	12	14	23
N^1-methylnicotinamide (mg./hr.)	0.30	0.30	0.21	0.38
Riboflavin (μg./hr.)	21	26	23	57
B. Excretion after load tests [2]				
Ascorbic acid (mg./4 hr.)	61	103	59	113
Thiamine (μg./4 hr.)	670	530	600	575
N^1-methylnicotinamide (mg./4 hr.)	7.25	6.55	5.25	4.20
Riboflavin (μg./4 hr.)	975	1860	1115	2195

[1] Analysis of variance shows P < 0.01 that the difference between the platoons during the 3 weeks is chance for all measurements in A.

[2] Analysis of variance shows P < 0.06 or more that the difference between the platoons during the 3 weeks is chance for all measurements in B.

tween platoons were demonstrated in the fasting rates of excretion of ascorbic acid, thiamine, riboflavin and N^1-methylnicotinamide. The results for the load tests showed far less statistical significance than those for the fasting specimens. There did tend to be mean differences between the platoons in the load tests for ascorbic acid, thiamine and riboflavin, but for N^1-methylnicotinamide the unsupplemented platoon showed less of a decrease than did the supplemented. This latter finding is not surprising in view of the known qualifications that must be placed on the interpretation of N^1-methylnicotinamide levels, owing to extreme individual variations in response to test doses of nicotinamide (Sargent, Robinson and Johnson, '44).

The fasting rate of excretion of thiamine, riboflavin and N^1methylnicotinamide has been widely used in the assessment of nutritional deficiencies (Najjar and Holt, '42), and the present observations suggest that even in well-fed young men these observations may be at least as reliable as some kinds of load test. We shall turn now to a comparison of fasting levels with postcibal.

C. Levels in blood and urine at different times of day

For these comparisons the subjects were twelve men isolated for 2 weeks on a barren, windswept island in Buzzard's Bay, Massachusetts. The weather was fine but windy and cool, with an overall range of temperature from $+ 43°F.$ to $+ 57°F.$ Activities included maintaining camp, conducting laboratory work, and engaging in moderate activity outdoors, with an estimated average daily work expenditure of about 3500 Cal.

They subsisted throughout on a field ration that provided a variety of components but essentially constant daily intake of nutrients as shown in table 3.

Samples of venous blood were drawn every morning immediately after reveille but before breakfast, and timed specimens of urine were obtained at the same period. Postprandial specimens were collected at other times of day, so that by the end of 12 days comparisons had been made between fasting levels and levels in midmorning, early afternoon, late afternoon and after supper.

1. Results for blood (table 4). Serum protein and chloride showed but slight changes, with a tendency to a diurnal increase. Hemoglobin remained almost unchanged except for a small unexplained decrease during the ninth day. Serum ascorbic acid showed increases which were small when expressed in absolute amounts, but were large on a percentage basis. In view of the relatively large intake of ascorbic

acid at the noon meal (table 3) the smallness of the diurnal change was somewhat surprising. The general conclusion for blood is that under the present circumstances assessment of hemoglobin, serum protein, serum chloride and serum ascorbic acid could be made satisfactorily from samples taken at any time of day.

TABLE 3

Average daily consumption of second group of test subjects.

	BREAKFAST 7 A.M.	DINNER 12 NOON	SUPPER 5:30 P.M.	TOTAL FOR WHOLE DAY
Total Calories	1700	1270	1680	4650
Protein (gm.)	54	11	64	129
Ascorbic acid (mg.)	0	70	6	76
Thiamine (mg.)	0.9	0.4	0.6	1.9
Riboflavin (mg.)	1.2	0.1	1.4	2.7
Niacin (mg.)	8.6	2.7	11.4	22.7

TABLE 4

Fasting levels in whole blood and serum compared with levels at other times of day (average of 12 subjects).

TEST DAY	SPECIMEN	HEMOGLOBIN	SERUM PROTEIN	SERUM CHLORIDE	SERUM ASCORBIC ACID
		gm./100 ml.	gm./100 ml.	meq./l.	mg./100 ml.
3	9 A.M.	15.5	6.5	105	.
5	Fasting, 7 A.M.	15.4	6.4	103	0.7
5	1 P.M.	15.3	6.5	104	0.9
5	Δ	− 0.1	+ 0.1	+ 1	+ 0.2
7	Fasting, 7 A.M.	15.4	6.8	102	0.5
7	3 P.M.	15.6	6.8	103	0.6
7	Δ	+ 0.2	0	+ 1	+ 0.1
9	Fasting, 7 A.M.	15.8	6.4	101	0.4
9	7:30 P.M.	15.2	6.8	102	0.6
9	Δ	− 0.6	+ 0.4	+ 1	+ 0.2

2. *Results for urine (table 5).* The urinary data should be examined with a view to obtaining from random specimens those values which are directly related to fasting levels. Preferably one would desire a reasonable approximation of the order of magnitude of ± 10% of the fasting value, but any constant relationship would satisfy the present requirements. However, neither expressing the results as concentration per 100 ml. of urine (table 5B), nor as concentration per 100 ml. of urine, corrected to specific gravity 1.010 (table 5C), nor as excretion per hour (table 5D) allowed chloride, ascorbic acid, thiamine, N^1-methylnicotinamide or riboflavin in random specimens to be calculated

MEASUREMENT	Day 3 F[1]	Day 3 11 A.M.	Day 3 Δ, %F[1]	Day 5 F[1]	Day 5 3 P.M.	Day 5 Δ, %F[1]	Day 7 F[1]	Day 7 9 P.M.	Day 7 Δ, %F[1]	Day 9 F[1]	Day 9 6 P.M.	Day 9 Δ, %F[1]
A. Rate of urinary flow and specific gravity of urine												
Urine volume (ml./hr.)	44	91	+ 107	36	92	+ 155	46	57	+ 24	44	99	+ 125
Urine specific gravity	1.026	1.021	− 0.5	1.026	1.017	− 0.9	1.026	1.023	− 0.3	1.023	1.024	+ 0.1
B. Excretion per 100 ml. urine												
Chloride (gm. NaCl/100 ml.)	1.20	1.17	− 3	1.20	0.70	− 42	1.06	1.02	− 4	1.23	1.05	− 15
Ascorbic acid (mg./100 ml.)	1.4	1.7	+ 21	1.7	1.6	− 6	1.1	1.6	+ 46	1.1	1.5	+ 36
Thiamine (μg./100 ml.)	9	9	0	8	9	+ 13	9	12	+ 33	11	8	− 27
N[1]-methylnicotinamide (mg./100 ml.)	0.80	0.75	− 6	1.10	0.60	− 45	0.45	0.60	+ 33	0.80	0.60	− 25
Riboflavin (μg./100 ml.)	82	33	− 60	86	27	− 69	62	56	− 10	55	30	− 45
C. Excretion per 100 ml. urine, calc. to Sp. G. 1.010												
Chloride (gm. NaCl/100 ml.; Sp.G. 1.010)	0.46	0.55	+ 20	0.46	0.41	− 11	0.41	0.44	+ 7	0.54	0.44	− 19
Ascorbic acid (mg./100 ml.; Sp.G. 1.010)	0.5	0.8	+ 60	0.7	0.9	+ 29	0.4	0.7	+ 75	0.5	0.6	+ 20
Thiamine (μg./100 ml.; Sp.G. 1.010)	3	4	+ 33	3	5	+ 67	3	5	+ 67	5	3	− 40
N[1]-methylnicotinamide (mg./100 ml.; Sp.G. 1.010)	0.30	0.35	+ 17	0.40	0.35	− 13	0.15	0.25	+ 67	0.35	0.25	− 29
Riboflavin (μg./100 ml.; Sp.G. 1.010)	32	16	− 50	33	15	− 55	24	25	+ 4	24	13	− 46
D. Excretion per hour												
Chloride (gm. NaCl/hr.)	0.53	1.06	+ 100	0.37	0.64	+ 73	0.49	0.58	+ 18	0.54	1.04	+ 93
Ascorbic acid (mg./hr.)	0.6	1.5	+ 150	0.5	1.5	+ 200	0.5	0.9	+ 80	0.5	1.5	+ 200
Thiamine (μg./hr.)	4	8	+ 100	3	8	+ 167	4	7	+ 75	5	8	+ 60
N[1]-methylnicotinamide (mg./hr.)	0.35	0.70	+ 100	0.35	0.55	+ 57	0.20	0.35	+ 75	0.35	0.60	+ 71
Riboflavin (μg./hr.)	36	30	− 17	27	25	− 7	29	32	+ 10	24	30	+ 25

[1] Represents specimens collected between the time of emptying bladder on arising and the time of breakfast.

so as to be even roughly comparable with fasting values. The nearest approaches were for chloride when expressed as concentration per 100 ml. urine (2 out of 4 values) and for riboflavin when expressed as excretion per hour (2 out of 4 values). The latter finding is at variance with the interpretation put on their data by Feder, Lewis and Alden ('44) who found much more constancy when urinary riboflavin was expressed in terms of actual concentration than when it was calculated on an hourly basis. Their experimental conditions and ours were so widely different that this discrepancy is not disturbing.

DISCUSSION

The present results are applicable to active young men subsisting on a relatively constant diet, such as field rations, and are not necessarily valid for other types of population or diets, particularly deficient ones. With these reservations in mind, we can draw three conclusions of practical importance in conducting nutrition surveys when chloride, ascorbic acid, thiamine, N^1-methylnicotinamide, riboflavin, hemoglobin and serum protein are in question. First, if it is not possible to conduct all the tests desired, preference should be given to obtaining fasting specimens rather than to conducting load tests. Second, under the same conditions and in temperate environments, specimens of blood give reasonably comparable results regardless of the time of day. Finally, it is highly desirable to collect fasting rather than random specimens of urine, owing to the difficulty in obtaining values otherwise even roughly comparable with fasting levels.

SUMMARY

1. Repetition of 4-hour oral vitamin loading tests employing ascorbic acid, thiamine, riboflavin and nicotinamide administered at intervals of 2 or 3 weeks to soldiers subsisting on good field rations had no significant effects on the results of subsequent loading tests or on the fasting urinary excretion of ascorbic acid, thiamine, riboflavin or N^1-methylnicotinamide.

2. Under the same circumstances the fasting rates of urinary excretion of ascorbic acid, thiamine, riboflavin and N^1-methylnicotinamide were more sensitive measures of vitamin intake in the previous 3 weeks than were 4-hour oral vitamin loading tests.

3. Whole blood hemoglobin serum protein, serum chloride and serum ascorbic acid varied relatively little in the course of the day.

4. In contrast to the results for blood and serum, the fasting urinary rates of excretion of chloride, ascorbic acid, thiamine, N^1-methylnicotin-

mide and riboflavin and values obtained at other times of day were ot satisfactorily related in a simple manner either when expressed: a) as concentration per 100 ml. of urine, (b) as concentration per 100 nl. of urine corrected to specific gravity 1.010, or (c) as excretion per our.

5. The practical conclusions for nutritional surveys are that when t is not possible to conduct all the tests that might be desired, fasting evels should be determined in preference to the results of loading tests; hat specimens of blood may with reservations be drawn at any time of lay; and that random specimens of postprandial urine should be ιvoided if possible. These conclusions are limited to hemoglobin, serum protein, chloride and ascorbic acid; and to urinary chloride, ascorbic ıcid, thiamine, N^1-methylnicotinamide and riboflavin.

ACKNOWLEDGMENTS

Operation of a field nutrition laboratory was made possible by the skilled technical assistance of T/4 J. Poulin, T/4 A. Razoyk, T/5 M. Castiglione, T/4 E. Ainsworth and Pfc. J. Stachelek, assigned to the Harvard Fatigue Laboratory by the Quartermaster's Climatic Research Laboratory, Lawrence, Mass.

We are indebted to Dr. L. A. Contreras of Lima, Peru, Fellow of the Institute of International Education, for his assistance in this work.

LITERATURE CITED

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. and Med., vol. 34, p. 146.

FEDER, V. H., G. T. LEWIS AND H. S. ALDEN 1944 Studies on the urinary excretion of riboflavin. J. Nutrition, vol. 27, p. 347.

HARVEY, S. C. 1910 The quantitative determination of chlorides in the urine. Arch. Int. Med., vol. 6, p. 12.

JOHNSON, R. E., L. A. CONTRERAS, P. F. ROBINSON AND F. C. CONSOLAZIO 1945 A comparison of intravenous and oral vitamin tolerance tests. In preparation.

JOHNSON, R. E., R. C. DARLING, F. SARGENT AND P. ROBINSON 1945 Effects of variations in dietary vitamin C on the physical well being of manual workers. J. Nutrition, vol. 29, p. 155.

JOHNSON, R. E., F. SARGENT, P. F. ROBINSON AND F. C. CONSOLAZIO 1945 Rapid field methods for the estimation of riboflavin, thiamine and N^1-methylnicotinamide. Ind. Eng. Chem. (Analytical Ed.), vol. 17, p. 384.

NAJJAR, V. A., AND L. E. HOLT, JR. 1942 A simple method for the laboratory diagnosis of subclinical deficiencies of thiamine, riboflavin and nicotinic acid. Bull. Johns Hopkins Hosp., vol. 70, p. 329.

PHILLIPS, R. A., D. D. VAN SLYKE, V. P. DOLE, K. EMERSON, JR., P. B. HAMILTON AND R. M. ARCHIBALD 1943 Copper sulfate method for measuring specific gravities of whole blood and plasma. Privately published.

SARGENT, F., P. F. ROBINSON AND R. E. JOHNSON 1944 F_1 and F_2 of Najjar and Holt in the urine of normal young men. J. Clin. Invest., vol. 23, p. 714.

EFFECTS OF FOOD INTAKE AND ANOXIA UPON ASCORBIC ACID EXCRETION, ACIDITY OF URINE, AND SURVIVAL OF MALE ALBINO RATS[1]

KATHERYN E. LANGWILL,[2] C. C. KING AND GRACE MACLEOD
Department of Chemistry, Columbia University and the Nutrition Laboratory of Teachers College, Columbia University, New York

(Received for publication March 15, 1945)

The present investigation was undertaken because of the need for experimental data concerning (a) the effect of food intake upon optimal resistance to anoxia and (b) the trend of reactions that are induced by lowered atmospheric pressure. Concurrent studies with human subjects gave additional evidence of the importance of food composition in relation to altitude tolerance.

Evidence has been presented by Packard ('07), Boothby et al. ('40), Polonovski ('40), McFarland ('41) and others, that a diet high in carbohydrates can afford measurable protection against anoxia. That carbohydrate metabolism in turn, is affected by exposure to anoxia has been demonstrated also (Van Middlesworth et al., '44; Bryan et al., '44; Warren, '41; McQuarrie et al., '42). Campbell ('38) concluded that the diet which gives greatest protection against acute anoxia in white rats is one made up solely of carrots. Many of his observations were confirmed by Nelson et al. ('43). Diets found by Campbell to give no protection against anoxia included raw and cooked horse meat, cheese, and whole milk, all of which are excellent sources of protein. Contrary to these findings, Bierman ('43) suggested, although without supporting experimental data, that a diet high in protein is preferable. There are sound theoretical reasons, however, for believing that preflight and inflight meals high in protein would impair altitude tolerance, and carefully controlled observations with human subjects have demonstrated there is such a relationship (King et al., '45; Butts, Mulholland and Green, '45).

[1] This investigation was aided by a grant from The Nutrition Foundation.

[2] The experimental data given in the present manuscript were presented by one of the authors (K. E. L.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, under the Joint Committee on Graduate Instruction, Columbia University.

EXPERIMENTAL

In this investigation three specific alterations in diet were studied.)ne group of rats was given a diet of Purina dog chow ad libitum and ι daily supplement of 5 gm. of raw horse meat. A control group was :ontinued on the dog chow diet alone. Second, two groups of rats were 'ed dog chow ad libitum, but a daily supplement of 2 gm. of sucrose in syrup form was fed to one group. Third, one group of rats was fed lehydrated carrots ad libitum as the sole source of food and the control group was fed an isocaloric quantity of dog chow. Before determining the relative survival rates when subjected to severe anoxia (3% of oxygen, 97% of nitrogen), composite urine samples were collected from each group during three periods for the determination of pH, titratable acidity, and ascorbic acid. During the first period, all animals were on the chow diet; during the second, one group was on the chow diet and the other received chow plus the supplement; during the third period, experimental diets were continued and the animals were subjected to mild anoxia (7.7% of oxygen, 92.3% of nitrogen).

Apparatus. To permit collection of composite urine samples, the animals were placed in long narrow cages divided into eight compartments, each of which accommodated two rats. The cages were equipped with stainless steel wire screen bottoms and were placed over glass-lined funnels. Mild anoxia (simulated altitude, 25,000 feet) was produced by exposing the animals to a gas mixture containing 7.7% of oxygen and 92.3% of nitrogen at atmospheric pressure.

Analytical procedure. On the days that ascorbic acid content of the urine was to be determined, the urine was collected in metaphosphoric acid of such strength that the final concentration was approximately 3%. The ascorbic acid content was then determined by use of a Coleman Universal Spectrophotometer, Model 11, following the procedure recommended by Bessey ('38) for the determination of small amounts of ascorbic acid in turbid solutions in the presence of other reducing substances.

Titratable acidity was determined on the composite urine samples according to Folin's method. A Beckman pH meter, Laboratory Model G, was used to determine pH values.

Experimental animals. Male albino rats from Professor Sherman's well standardized colony were used. Any animals that did not show a normal gain in weight after being weaned (28 days) and placed on a regimen of Purina dog chow (proximate analysis: carbohydrate 48.5, protein 26.2, fat 5.4%) and tap water ad libitum were discarded.

Protein tests. (A) Fifteen pairs (litter-mates) of male albino rats were divided into two groups in such a way that the average age of the rats in each group was 88 days and the average body weight was 210 gm. For a period approximately 2 weeks, both groups were continued on the chow diet. Composite urine samples were collected over a 3-hour period, during which time the animals did not have access to either food or water. Samples for pH and titratable acidity measurements and for ascorbic acid determinations were collected on successive days.

TABLE 1

Results (averages) obtained during three successive periods of two protein feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on meat supplement; (3) all animals subjected to mild anoxia.

	ASCORBIC ACID				ACID-BASE DATA					
	Volume				Volume		pH		N/10 NaOH per ml.	
	Groups		Groups		Groups		Groups		Groups	
	I	II	I	II	I	II	I	II	I	II
	ml.		*µg./ml.*		*ml.*		*Range*		*ml.*	
A. First series:										
1st period (3 hrs.)	13.2	12.8	37	45	11.1	11.6	6.3–7.0	6.6–7.7	0.44	0.30
2nd period (3 hrs.)	15.1	25.8	56	51	13.9	24.0	6.1–6.7	6.6–7.1	0.56	0.39
3rd period (2 hrs.)	44.1	52.5	10	6	48.1	55.6	7.2–7.6	7.4–7.8	0.10	0.06
B. Second series:										
1st period (6 hrs.)	12.4	11.1	38	37	12.1	11.3	5.3–7.5	5.6–7.9	0.36	0.34
2nd period (6 hrs.)	13.2	20.6	31	21	14.7	20.5	5.4–7.0	6.1–7.5	0.57	0.34
3rd period (3 hrs.)	35.5	52.4	4	2	32.4	49.5	8.1–8.3	8.6–8.6	0.00	0.00

During a 16-day period, the diet of group I was unaltered, and group II was given a supplement of 5 gm. of raw horse meat half an hour before the collection of the composite urine sample. Both groups were subjected to mild anoxia during the following week. After obtaining the ascorbic acid, pH and titratable acidity values on 3 different days, the animals were subjected to severe anoxia (5% oxygen, 95% nitrogen). Six of the chow-fed group and two of the meat-fed group survived this treatment. In table 1 will be found the average results of analysis of six composite urine samples collected during the preliminary and supplement-feeding periods and of three samples collected during the period of mild anoxia. The average body weights of the control and meat-fed groups were, respectively: 210 and 210 gm. at the beginning, 232 and 231 gm. when the supplement was started; and 254 and 264 gm. at the end.

(B) Because of the wide variations in results obtained by analysis of composite urine samples collected while the animals were held under comparable conditions, the experiment outlined in (A) was repeated, with slight changes. Urine samples were collected from sixteen individual male rats (average age, 86 days; average weight, 154 gm.) over a 6-hour interval. The samples were titrated with 2,6-dichlorophenol indophenol to an endpoint which lasted approximately 5 seconds. The sixteen rats were then divided into two groups in such a way that the average ascorbic acid excretion per milliliter of urine was approximately the same for each group. During the next 2-week period, a supplement of 5 gm. of raw horse meat was fed daily (with the exception of Saturdays and Sundays) to each animal in group II. Urine samples from the individual rats were collected during the period immediately after consumption of the meat. Titratable acidity and pH, and ascorbic acid determinations were made on alternate days. Composite urine samples were collected during a 3-hour exposure to mild anoxia. Average values are given in the B section of table 1 for the individual samples collected during 4 days in the first and second periods of the experiment and on the composite samples collected during the period of mild anoxia. The average initial, intermediate and final body weights of the animals in groups I and II were, respectively: 156 and 151 gm.; 169 and 172 gm.; and 204 and 229 gm. The two groups of rats were then subjected to severe anoxia for an hour and three-quarters. Of the meat-fed group, one survived; of the chow-fed group, four survived.

Carbohydrate tests. (A) Thirty male albino rats, average age 111 days and body weight 248 gm., were divided into two groups of fifteen each. During the preliminary period, 3-hour composite urine samples were collected from each group and analyzed. The animals in one group were then given, by means of a medicine dropper, 2 gm. of sucrose in syrup form (66.6% by weight) each day immediately before collecting the 3-hour urine sample (second period). Both groups of rats were then subjected to mild anoxia for 2 hours (third period). One rat in the chow-fed group and four in the sugar-fed group did not survive this treatment. The 2-hour exposures to mild anoxia and supplement feeding were continued, and the data obtained on ascorbic acid and titratable acidity were calculated to a basis of fifteen rats in each group. When the two groups were subjected to severe anoxia, five rats in each group survived. The average values obtained on analysis (a) of six samples of urine collected during the preliminary and supplement-feeding periods and (b) of five samples collected during the period

of anoxia are given in table 2. The intermediate and final average body weights of groups I and II were 251 and 249, and 273 and 265 gm., respectively.

TABLE 2

Results (averages) obtained during three successive periods of three series of carbohydrate feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on sugar supplement; (3) all animals subjected to mild anoxia.

		Ascorbic acid					Acid-base data				
		Volume		Concn.		Volume		pH		N/10 NaOH per ml.	
		Groups		Groups		Groups		Groups		Groups	
		I	II	I	II	I	II	I	II	I	II
		ml.		*µg./ml.*		*ml.*		*Range*		*ml.*	
				A. First series:							
1st period	(3 hrs.)	14.1	14.6	31	48	14.5	15.6	6.1–7.0	6.3–7.4	0.52	0.46
2nd period	(3 hrs.)	14.8	12.3	34	77	15.1	12.2	6.3–6.7	6.0–6.2	0.49	0.60
3rd period	(2 hrs.)	62.7	64.5	7	15	53.2	51.1	6.9–7.5	7.0–7.6	0.09	0.09
				B. Second series:							
1st period	(4 hrs.)	16.7	16.3	87	86	15.7	15.3	6.0–6.3	6.2–6.5	0.69	0.62
2nd period	(4 hrs.)	15.1	14.5	80	86	16.4	16.3	6.1–6.4	6.2–6.6	0.55	0.45
3rd period	(2 hrs.)	39.4	65.1	20	12	22.3	34.1	7.6–7.7	8.0–8.2	0.05	0.00
				C. Third series:							
1st period	(6 hrs.)	16.1	17.3	39	37	15.8	15.4	5.7–7.6	5.5–6.7	0.49	0.54
2nd period	(6 hrs.)	16.1	20.3	37	27	16.4	18.1	5.8–8.1	5.6–7.2	0.37	0.32
3rd period	(3 hrs.)	47.7	47.1	3	1	44.5	51.2	8.5–8.6	8.6–8.6	0.00	0.00

(B) Twenty-eight male albino rats were divided into two groups of fourteen animals each, average age 138 days, average body weight 255 gm. in group I and 263 gm. in group II. The animals were subjected to essentially the same treatment as before. The average values for ascorbic acid, pH, and titratable acidity are recorded in table 2. The average body weights in groups I and II were 255 and 264 gm., respectively. At the conclusion of the experiment when severe anoxia was administered, eleven animals in the chow-fed group and eight in the sugar-fed group survived.

(C) Twenty male albino rats were separated into two groups of ten each; the average initial ages and body weights of animals in groups I and II were 82 and 78 days, and 182 and 186 gm., respectively; average final body weights in groups I and II were 236 and 242 gm. The average values for individual samples collected on 4 days during the preliminary and supplement-feeding (2 gm. of sucrose) periods are given in table 2 along with the averages of four composite samples collected during the period of anoxia.

Carrot tests. (A) Fifteen pairs (litter-mates) of male albino rats were separated into two groups, average age 105 days, average body

weight 217 gm. The chow diet was continued for both groups during the preliminary period when analyses were made on composite 3-hour samples. The animals of the two groups were next paired according to weight and placed in separate cages. One animal of each pair was given dehydrated carrots ad libitum and the other was fed an isocaloric amount of the chow. During the next 2 weeks, composite 3-hour samples were collected and analyzed. Mild anoxia was administered for only 2 days. When subjected to severe anoxia, nine rats in each group survived.

TABLE 3

Results (averages) obtained during successive periods of carrot feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on dehydrated carrots; (3) all animals subjected to mild anoxia, in series A.

	Ascorbic acid				Acid-base data					
	Volume		Concn.		Volume		pH		N/10 NaOH per ml.	
	Groups		Groups		Groups		Groups		Groups	
	I	II	I	II	I	II	I	II	I	II
	ml.		*µg /ml.*		*ml.*		*Range*		*ml.*	
A. First series:										
1st period (3 hrs.)	14.0	13.4	23	24	13.9	13.5	5.4–6.8	6.0–6.7	0.55	0.56
2nd period (3 hrs.)	11.8	15.2	20	5	12.0	14.5	6.2–6.5	8.1–8.3	0.66	0.00
3rd period (2 hrs.)	21.8	18.0	10	3	27.0	23.5	7.08	8.32	0.08	0.00
B. Second series:										
1st period (8 hrs.)	28.3	27.2	32	35	27.9	28.2	5.5–7.8	5.5–7.4	0.36	0.46
2nd period (3 hrs.)	8.1	13.5	38	2	10.3	13.0	6.0–6.1	8.3–8.5	0.47	0.00

The average values from six samples of urine obtained during the first period and five samples during the second period are reported in table 3. The samples were obtained from individual rats during the preliminary and carrot-feeding period but composite samples were necessary during the period of anoxia.

(B) During the preliminary period in which all the animals received the chow diet, individual samples of urine were collected from thirty-two male albino rats and analyzed as before. Two groups of sixteen rats each were then selected so that each rat had a litter-mate in the other group (average age 59 days, average body weight 102 gm.). One group was fed dehydrated carrots ad libitum and the other was allowed to consume an isocaloric quantity of chow, through a period of 9 days. The average values obtained for five samples during the preliminary period and two samples during the carrot-feeding period are given in table 3. The animals were not subjected to mild anoxia, but were exposed to severe anoxia immediately. Thirteen animals in each group survived.

(C) When dehydrated carrots were fed as the sole source of food, a state of alkalosis developed in the rats, urinary pH values of 8.0 and over being observed regularly. Considering that the alkaline state per se might increase the rats' tolerance of anoxia, twenty male albino rats were divided into two groups of ten each; one group was put on the carrot diet for 3 days and the other was continued on the chow diet ad libitum. The pH of the urine was determined the day before feeding the dehydrated carrots and 2 days thereafter. Severe anoxia was administered on the third day. Two animals in the chow-fed group and six in the carrot-fed group survived.

(D) The experiment was repeated using ten male albino rats in each group, but instead of using air diluted with nitrogen, the animals were subjected to reduced atmospheric pressure in a decompression chamber. Urinary pH values were of the same order as before. They were taken in 6 minutes to a pressure corresponding to 40,000 feet elevation and held there for 2 minutes. Three animals of the chow-fed group and four of the carrot-fed group survived this treatment. Since the rapidity of "ascent" in this experiment might have mitigated against the demonstration of a distinct difference in the survival capacity of animals, a third experiment was conducted.

(E) The feeding of carrots to one group (15 animals in each) was continued for 3 days. Each day both groups were subjected to anoxia in a decompression chamber. On the first day they were taken to a pressure corresponding to 20,000 feet elevation in 30 minutes and held there for 30 minutes. The next day the animals were taken to the same altitude in 30 minutes and held there for 1 hour. On the third day the pressure was decreased to 40,000 feet elevation in 30 minutes and held there for 2 hours. (Rats have a remarkable capacity to adjust to high altitudes, upon repeated exposures.) Composite urine samples were collected on all 3 days for pH determination. All animals on the carrot diet survived exposure on the last day, but only five animals on the chow diet survived. Urinary pH values for these three experiments are given in table 4.

TABLE 4

Effect of carrot feeding upon survival and urinary pH values.

	EXPERIMENT C		EXPERIMENT D		EXPERIMENT E	
	Chow-fed	Carrot-fed	Chow-fed	Carrot-fed	Chow-fed	Carrot-fed
Before carrot feeding	7.2	7.6	7.9	7.9	..	..
1st day of carrot feeding	7.0	8.6	6.3	8.3	7.1	8.0
2nd day of carrot feeding	7.2	8.6	7.0	8.3	6.6	8.2
Survival after severe exposure	2 of 10	6 of 10	3 of 10	4 of 10	5 of 15	15 of 15

DISCUSSION

An effect of anoxia which was apparent throughout these experiments, regardless of the diet or supplement fed, was polyuria. A similar observation has been reported by Silvette ('42) who also worked with male albino rats. He found that the urinary excretion per 100 gm. of body weight was increased over 300% when the animals were maintained for 3 hours at a simulated altitude of 15,000 feet in a low pressure chamber. In the present tests, volume increases of 100 to 300% were observed frequently.

Another effect was that of an increase in the pH with accompanying decrease in titratable acidity when the animals were subjected to mild anoxia, confirming the findings of Brassfield and Behrmann ('41), with dogs. A decrease in the ascorbic acid concentration occurred concomitantly with an increase in pH, irrespective of the diet or supplement fed. The phenomenon of a decrease in ascorbic acid content of alkaline urine has been observed by Hawley et al. ('36; '37). In the experiments in which dehydrated carrots were fed as the sole source of food and in those in which 5 gm. of raw horse meat were fed as a supplement to the chow diet, the decrease in ascorbic acid per milliliter of urine was noted before anoxia was induced. In the protein tests, a decrease in titratable acidity accompanied by a slight increase in pH was noted when the rats received the meat supplement. In rats the ratio of urea to ammonium nitrogen is approximately 8:1 (Griffith and Farris, '42), in contrast to a ratio of 25:1 (Best and Taylor, '43), observed in human urine.

Krasno et al. ('43) have studied the effect of exposure of human subjects to moderate anoxia, relative to the excretion of ascorbic acid. In forty-nine tests made on nineteen subjects, the urinary concentration of ascorbic acid was diminished in all but seven cases. In every case where urinary pH values were recorded before and after exposure to various altitudes up to 18,000 feet, the pH had increased in all but four tests. The pH in these four cases was 7.5 both before and after exposure.

A study of the effect of organic compounds which function as nerve depressants on the synthesis of ascorbic acid in the rat has shown (Longenecker et al., '40) that even though the substances differed greatly in chemical structure, the synthesis of ascorbic acid was increased enormously, sometimes as much as a hundredfold. Among the most active compounds were chloretone, paraldehyde, sodium phenobarbital, and calcium ipral. It was postulated tht the accelerated ascorbic acid synthesis is a protective mechanism available to the animal against foreign toxic substances, including those that suppress respira-

tion of the central nervous system, although there was no direct evidence of the mechanism involved. On the basis postulated, anoxia imposed by low oxygen tension would be analogous in some respects. Gordon et al. ('44) and Leblond ('44) observed that a diet to which thiourea (e.g., 0.5%) was added increased the altitude tolerance of rats. They were of the opinion that an induced state of functional hypothyroidism resulted in a reduction of the basal metabolic rate and hence an increased altitude tolerance. A starvation diet or one which causes loss in body weight may also reduce the basal metabolic rate. Smith et al. ('44) observed that cats given a diet (canned rabbit meat plus vitamin B supplements) restricted in amount so that they lost 15 to 20% of their body weights within 1 or 2 weeks showed an increase in resistance to low oxygen tension.

In the early carrot tests when the food consumption of rats on the chow diet was regulated to equal that of the group receiving dehydrated carrots, there was little difference in the tolerance of the two groups when subjected to a lethal degree of anoxia. All groups of animals reported in table 3 lost more than 10% of their body weight in 3 weeks.

It is evident that the degree of protection afforded against anoxia by means of carrot feeding varies markedly with the conditions of testing. The alkalinity of the urine of rats fed dehydrated carrots only is apparently caused chiefly by the high potassium content, without an equivalent amount of "fixed acids." Follis et al. ('42) have found that pathological alterations in the heart and kidneys of rats may be produced by feeding a diet extremely low in potassium content (0.01%). Possibly an excess of potassium affords a moderate degree of protection against anoxia. According to Dixon ('40) it is probable that nerve cells and fibers are impermeable to sodium and to the majority of anions, whereas potassium ions can penetrate the neuronal surfaces. From studies on the cerebral cortex in vitro, he found that deprivation of oxygen increases both the destruction of glucose and the production of lactic acid. The addition of excess potassium ions, however, markedly inhibited lactic acid production and glucose destruction. With this depression of metabolism, decreased oxygen consumption probably would follow and aid the animals in combating oxygen want.

SUMMARY

The effect of anoxia, induced by breathing a gas mixture low in oxygen at atmospheric pressure, on groups of male albino rats fed (a) dog chow alone, (b) a chow diet plus a daily supplement of 5 gm. of raw horse meat, (c) a chow diet plus a pre-exposure supplement of 2 gm. of

sucrose, and (d) dehydrated carrots as the sole source of food, has been studied relative to the ascorbic acid, pH, and titratable acidity of the urine and to survival.

In all experiments anoxia caused an increase in the pH of the urine and a decrease in titratable acidity, which was accompanied by polyuria.

The ascorbic acid concentration of the urine varied roughly with the pH value over wide ranges, a high pH being accompanied by a low ascorbic acid value.

Increased alkalinity of the urine of animals fed a horse meat supplement, compared with animals fed chow alone, was not accompanied by protection of the animals against marginal lethal exposures to anoxia. Altitude tolerance was in fact slightly less in the meat-fed (high protein intake) groups.

Under the conditions of this experiment, a pre-exposure sugar supplement did not affect the altitude tolerance of rats on a chow diet.

When the consumption of chow was regulated equicalorically with the consumption of dehydrated carrots fed ad libitum, the two groups of animals survived severe anoxia almost equally well under some conditions of testing but in other tests, with an opportunity for adaptation by repeated exposures and more gradual ascent, there was evidence of added protection as a result of the carrot diet. The increased alkalinity, high potassium content and low protein content of the carrot diet afford a tentative basis of accounting for the increased tolerance.

LITERATURE CITED

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., vol. 126, p. 771.

BEST, C. H., AND N. B. TAYLOR 1943 Physiological basis of medical practice. Third edition, Williams and Wilkins, Baltimore, p. 660.

BIERMAN, H. R. 1943 Nutrition in aviation medicine. War Med., vol. 3, p. 1.

BOOTHBY, WILLIAM M., W. R. LOVELACE, II AND O. O. BENSON 1940 High altitude and its effect on the human body. J. Aeronautical Sci., vol. 7, p. 461.

BRASSFIELD, C. R., AND V. G. BEHRMANN 1941 A correlation of the pH of arterial blood and urine as affected by changes in pulmonary ventilation. Am. J. Physiol., vol. 132, p. 272.

BRYAN, A. N., AND H. T. RICKETS 1944 The effect in man of chronic intermittent anoxia on glucose tolerance (acid urinary excretions). J. Clin. Endocrin., vol. 4, p. 450.

BUTTS, J. S., H. F. MULHOLLAND AND D. M. GREEN 1945 The relationship of anoxia susceptibility to diet. J. Aviation Med., in press.

CAMPBELL, J. A. 1938 Increase of resistance to oxygen want in rats on a carrot diet. J. Physiol., vol. 93, p. 31.

DIXON, K. C. 1940 A study of cortical metabolism in relation to cerebral disease. Brain, vol. 63, p. 191.

FOLLIS, R. H., JR., E. ORENT-KEILES AND E. V. MCCOLLUM 1942 The production of cardiac and renal lesions in rats by a diet extremely deficient in calcium. Am. J. Path., vol. 18, p. 29.

GORDON, A. S., E. D. GOLDSMITH AND H. A. CHARIPPER 1944 Thiourea and resistance to low atmospheric pressures. Science, vol. 99, p. 104.

GRIFFITH, J. Q., JR., AND E. J. FARRIS 1942 The rat in laboratory investigation. Lippincott, Philadelphia, p. 184.

HAWLEY, E. E., R. G. DAGGS AND D. J. STEPHENS 1937 The effect of administration of acid and alkaline salts upon ascorbic acid content of guinea pig tissue. J. Nutrition, vol. 14, p. 1.

HAWLEY, E. E., J. P. FRAZER, L. L. BUTTON AND D. J. STEPHENS 1936 Effect of administration of sodium bicarbonate and ammonium chloride on the amount of ascorbic acid found in the urine. J. Nutrition, vol. 12, p. 215.

HOFF, E. C., AND J. F. FULTON 1942 (and supplement, 1944) A bibliography of aviation medicine. Thomas, Springfield.

KING, C. G., H. F. BICKERMAN, C. J. HARRER, J. R. OYLER AND C. P. SEITZ 1945 Aviation nutrition studies. The effects of preflight and inflight meals of varying composition with respect to carbohydrate, protein and fat. J. Aviation Med., vol. 16, p. 69.

KRASNO, L. R., A. C. IVY, A. J. ATKINSON AND W. B. JOHNSON 1943 Effect of exposure to anoxia of a moderate degree on the excretion of ascorbic acid in the urine. War Med., vol. 3, p. 256.

LEBLOND, C. P. 1944 Increased resistance to anoxia after thyroidectomy and after treatment with thiourea. Proc. Soc. Exp. Biol. and Med., vol. 55, p. 114.

LONGENECKER, H. E., H. H. FRICKE AND C. G. KING 1940 The effect of organic compounds upon vitamin C synthesis in the rat. J. Biol. Chem., vol. 135, p. 497.

MCFARLAND, R. A. 1941 Fatigue in aircraft pilots. New Eng. J. Med., vol. 225, p. 845.

MCQUARRIE, I., M. R. ZIEGLER AND L. J. HAY 1942 The effect of anoxia on blood sugar in adrenalectomized dogs. Proc. Soc. Exp. Biol. and Med., vol. 49, p. 291.

NELSON, D., S. GOETZL, S. ROBINS AND A. C. IVY 1943 Carrot diet and susceptibility to acute anoxia. Proc. Soc. Exp. Biol. and Med., vol. 52, p. 1.

PACKARD, W. H. 1907 The effect of carbohydrates on resistance to lack of oxygen. Am. J. Physiol., vol. 18, p. 164.

POLONOVSKI, M. 1940 Effect of sugar ingestion on resistance to anoxemia and acapnia at high altitudes. Bull. Acad. Med., vol. 123, p. 688.

SILVETTE, H. 1942 Influence of repeated daily exposure to low barometric pressure on urine output. Proc. Soc. Exp. Biol. and Med., vol. 51, p. 199.

SMITH, D. C., R. H. OSTER AND J. E. P. TOMAN 1944 The effect of thiamine deficiency and of reduced food intake on resistance to low oxygen tension in the cat. Am. J. Physiol., vol. 140, p. 603.

VAN MIDDLESWORTH, L., R. F. KLINE AND W. BRITTON 1944 Carbohydrate regulation under severe anoxia conditions in rats. Am. J. Physiol., vol. 140, p. 474.

WARREN, C. O. 1941 Respiration and glycogenolysis of bone marrow of rabbits exposed to lowered oxygen tension. Am. J. Physiol., vol. 135, p. 249.

STUDIES ON THE PROLONGED MAINTENANCE OF ADULT DOGS ON PURIFIED DIETS

A. O. SEELER AND R. H. SILBER
Merck Institute for Therapeutic Research, Rahway, New Jersey

(Received for publication May 3, 1945)

It is now well established that thiamine, riboflavin, nicotinic acid and pyridoxine are required by the adult dog. There are, however, few data in the literature concerning the importance of pantothenic acid, choline, inositol, biotin and other B complex factors for the nutrition of the adult dog. In this communication observations are reported on adult dogs maintained for about 4½ years on a basal diet which, according to present knowledge, had no significant amounts of the members of the B vitamin complex but was supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine and in some cases pantothenic acid.

Six mongrel female dogs, 1 to 1½ years old, were placed on test in July, 1940, after having been immunized against distemper, dewormed, and found to be normal both by physical examination and by study of the blood and the urine. The percentage composition of the basal diet (620) was as follows: casein "vitamin-free"[1] 30; dextrose 41; hydrogenated cottonseed oil[2] 21; corn oil 0.15; salt mixture U.S.P. XI 3; bone ash 2.85; and cod liver oil 2. Since, at the outset of the experiment, the dogs were to be prepared for studies on pantothenic acid deficiency, the basal diet was supplemented in all six animals by daily doses of 1 mg. each of thiamine, riboflavin, and pyridoxine and 10 mg. of nicotinic acid. Two dogs were given, in addition, 10 mg. of calcium pantothenate. Adequate amounts of vitamins A and D were supplied by the cod liver oil in the basal diet. The dogs were given 20 mg. of alpha tocopherol once a week.

For the first 6 months all the dogs appeared normal in spite of the fact that within the first 3 months their urinary excretion of pantothenic acid had dropped from a range of 100–500 μg. per dog per day to 2–15 μg. per dog per day. However, beginning in the seventh month of the experiment, one of the dogs on the pantothenic acid deficient diet began to appear rather listless and started to lose weight. One morning, just

[1] Casein Harris was used until September, 1943, when Casein Labco was substituted.

[2] Crisco.

9 months after the experiment was begun, she was found dead in her cage. This sudden death came as a surprise as on examination on the previous day the dog, while listless and somewhat emaciated, seemed otherwise normal. As shown in table 1, this dog (111) had a microcytic hypochromic anemia at the time of death. On autopsy the liver was found to be fatty but the other tissues showed no gross pathological changes.

The other three dogs on the pantothenic acid deficient diet showed no gross evidence of disease until the beginning of the second year on the diet when two of them (103 and 113) became listless and developed anorexia with associated weight loss. At the time the weight loss started the dogs also began to show a gradual decline in erythrocyte count and hemoglobin. Because of the loss of appetite it was thought that the anemia might be due to iron deficiency. Accordingly the animals were given 250 mg. of ferrous sulfate daily in solution by stomach tube. By the seventeenth month the two dogs had lost a great deal of weight and were very weak. One of the two had developed indolent ulcers at pressure points. Both dogs showed a rather coarse coat and there was some depigmentation of the black spots in the coats of both dogs. Otherwise physical examination revealed no abnormality. The anemia had progressed in spite of the iron therapy; the lowest values obtained are shown in table 1. Studies on the blood chemistry of

TABLE 1

Hemoglobin and erythrocyte values.

DOG	DATE	ERYTHROCYTES MILLIONS	HEMATOCRIT	HEMOGLOBIN GRAMS %	COLOR INDEX	M.C.V.[1]	M.C.H.[2]
			Control values				
76	7- 9-'40	6.48	43.5	12.5	0.66	67.1	19.3
103	7-12-'40	7.60	45.0	14.1	0.64	59.2	18.6
111	7-18-'40	8.12	49.5	14.4	0.61	61.0	17.7
112	7-18-'40	7.79	47.5	14.1	0.62	61.0	18.1
113	7-12-'40	7.25	41.0	11.4	0.54	56.6	15.7
			At time of acute pantothenic acid deficiency				
103	12- 9-'41	4.70	30.0	8.2	0.60	63.8	17.4
111	4-18-'41	6.70	33.0	9.5	0.49	49.3	14.2
113	12- 9-'41	6.32	39.0	9.5	0.52	61.7	15.0
			After 4½ years on purified diet				
76	11-28-'44	6.06	43.5	12.3	0.70	71.8	20.3
103	11-28-'44	6.13	42.0	11.9	0.67	68.5	19.4
112	11-28-'44	7.31	49.0	14.5	0.68	67.0	19.8

[1] Mean corpuscular volume. [2] Mean corpuscular hemoglobin.

these dogs were made by Scudi and Hamlin ('42) and have been reported. They found that the total lipoid and the cholesterol concentrations of the blood of the two dogs were lower than those of the controls. There was no increase in the nonprotein nitrogen content of the blood, and fasting blood glucose values were normal.

In order to establish the causal relationship of pantothenic acid to this syndrome, both dogs were given 50 mg. of calcium pantothenate by mouth for 3 days and were then given 10 mg. daily. Within 2 days after the vitamin was administered there was slight, but definite improvement in the condition of the animals. After 1 month the dogs were active and had regained most of the weight loss. The indolent ulcers noted in one of the animals had healed. Their coats were in good condition and the normal pigmentation was being restored. The blood lipoids had returned to normal. There had been no striking reticulocyte response following the administration of pantothenic acid, but there was a gradual increase in both erythrocyte count and hemoglobin, although the values did not return to normal until after 3 months of pantothenic acid therapy.

At the time this report is written, $4\frac{1}{2}$ years after the onset of the experiment, three of the original six dogs are still living. Of the three that died one was found dead after 9 months on a pantothenic acid free diet, but the other two unfortunately died by accident. One of the surviving dogs has had no pantothenic acid since the beginning of the experiment, while the second has received pantothenic acid in her supplement throughout the experiment; the third, while on a pantothenic acid free diet for the first 17 months, has been receiving pantothenic acid since then.

The three surviving dogs (76, 103 and 112) are active and alert. Their appetite is good and their weights are about the same as at the beginning of the experiment. The coats are normal in texture and pigmentation, and the skin and mucous membranes are in good condition. Neurological examination reveals no abnormalities. Slit lamp examination was not carried out, but opthalmoscopic examination of the eyes shows no pathological changes.

The blood pictures are normal as is shown in table 1. Except for an increased excretion of indican, the significance of which is not known, urinalyses gave normal results. Serum protein and serum chloride determinations gave normal values. The values for fibrinogen, blood, urea, and non-protein nitrogen were within the normal range. A study of the ability of the kidneys to excrete a concentrated or dilute urine showed no impairment. Gastric analyses showed both the free and

total acidity to be normal. All three dogs were found to show some increase in bromsulfalein retention, suggesting an impairment in hepatic function. It is of interest that the increased bromsulfalein retention was first observed in these dogs at the end of the first year of the experiment and has grown no worse.

DISCUSSION

The observations on adult dogs on a pantothenic acid deficient diet indicate that the dietary requirement for this vitamin is not critical since many months may elapse before deficiency becomes manifest. It is impossible to predict the time required to deplete an adult animal, as in this experiment one of four dogs died after 9 months presumably of pantothenic acid deficiency; two were cured by pantothenic acid when they seemed on the verge of death after 17 months on test, while the fourth still shows no signs of deficiency in spite of the fact that she has been given no pantothenic acid for 4½ years. While it is possible that some coprophagy occurred, it could not have been great as the dogs were kept on a coarse mesh screen which was washed daily. The basal diet was shown to have a maximum of 0.1 μg. of pantothenic acid per gram by microbiological assay, and when given to young rats, the syndrome of pantothenic acid deficiency appeared at the expected time. Furthermore, a group of four weanling puppies fed the same diet and supplement all died within 6 weeks, whereas, two litter mate controls on this diet but receiving a supplement containing pantothenic acid were still apparently healthy after 6 weeks. This confirms the report of Schaefer, McKibbin and Elvehjem ('42) who found that the requirement of growing dogs for pantothenic acid was much greater than that of the adult dog. Unna and Richards ('42) have reported that the adult rat requires less pantothenic acid than the young animal.

The clinical picture of pantothenic acid deficiency in the adult dog has no striking features which can be regarded as pathognomonic. Laboratory studies might be of some value in differential diagnosis in that if a debilitated dog shows a moderate anemia, low blood total lipoid and cholesterol values, together with the low pantothenic acid blood levels and urinary excretion values that have been reported by Silver ('44), a diagnosis of pantothenic acid deficiency is suggested, but a therapeutic trial would be necessary to prove the diagnosis.

Under the conditions of this experiment, the lack of inositol, biotin, "folic acid" and perhaps other unidentified B complex factors in the diet did not result in any evidence of deficiency disease in the adult dog over a period of 4½ years with the exception of some increase in

bromsulfalein retention, suggesting the possibility of moderate liver damage. While choline was not given per se, the diet contained 30% casein and consequently had a relatively large amount of methionine.

These observations on the adult dogs differ from the results of studies on puppies reported by Schaefer, McKibbin and Elvehjem ('42) who found that the addition of thiamine, riboflavin, pyridoxine, nicotinic acid and choline to a B complex free ration containing 19% casein did not suffice to maintain the puppies in good health. They found that the resulting condition was curable by liver extract but felt on the basis of preliminary data that mixtures of inositol, p-aminobenzoic acid and glutamine were not curative. Lambooy and Nasset ('43) found that puppies on a vitamin free ration containing 35% casein supplemented with all the synthetic members of the B complex except biotin failed to survive unless liver or yeast was added. Fouts ('43) found that puppies maintained on a vitamin free high protein (41.4% casein) basal diet supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline did very well, whereas, puppies on a lower protein (15% casein) diet died within 88–267 days.

If, as is suggested by the experiment of Fouts, the percentage of protein is a critical factor in the maintenance of dogs on an artificial diet these experiments on adult dogs cannot justly be compared with the experiments on puppies cited above since the protein content of the diets was not identical. However, the fact that Lambooy and Nasset's puppies did not survive on a diet containing 35% casein, whereas the adult dogs apparently did so well on 30% casein, suggests that the dietary requirement of the adult dog is not as great as that of the growing dog for one or more of the following factors: choline, inositol, biotin, "folic acid" and other unidentified B complex factors. This decrease in dietary vitamin requirements may be due to an actual lowering of the metabolic requirements for the vitamins, an increased intestinal synthesis or a combination of both factors.

SUMMARY

1. The dietary requirement of the adult dog for pantothenic acid is so small that the production of pantothenic acid deficiency is a very slow process. Of four dogs maintained on a diet lacking pantothenic acid, one dog died in 9 months; two developed acute deficiency disease in 17 months and were cured by pantothenic acid, and the fourth dog showed no gross evidence of deficiency disease after 4½ years.

2. Three dogs have been maintained in apparent good health for 4½ years on a basal diet, supplemented with thiamine, riboflavin, nico-

tinic acid, pyridoxine and for two of the three dogs with pantothenic acid also. The basal diet according to present knowledge had no significant amounts of the members of the B vitamin complex.

ACKNOWLEDGMENT

The authors express their appreciation to Mr. Joseph O'Leary for valuable technical assistance.

LITERATURE CITED

FOUTS, PAUL J. 1943 Vitamin B complex studies in dogs: Production of cirrhosis of liver. J. Nutrition, vol. 25, p. 217.

LAMBOOY, J. P., AND E. S. NASSET 1943 The inadequacy of eight synthetic B vitamins for the nutrition of puppies — unknown factor (factors) in yeast and probably liver. J. Nutrition, vol. 26, p. 293.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Pantothenic acid deficiency studies in dogs. J. Biol. Chem., vol. 143, p. 321.

——— 1942 Studies on the vitamin B complex in the nutrition of the dog. J. Nutrition, vol. 23, p. 491.

SCUDI, J. V., AND MARGARET HAMLIN 1942 The effect of pantothenic acid deficiency on the blood lipoids of the dog. J. Nutrition, vol. 24, p. 273.

SILBER, ROBERT H. 1944 Studies of pantothenic acid deficiency in dogs. J. Nutrition, vol. 27, p. 425.

UNNA, K., AND G. V. RICHARDS 1942 Relationship between pantothenic acid requirement and age in the rat. J. Nutrition, vol. 23, p. 543.

THE AVAILABILITY OF SOYBEAN OIL MEAL PHOSPHORUS FOR THE RAT [1]

ROBERT R. SPITZER AND PAUL H. PHILLIPS
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

(Received for publication February 16, 1945)

There is considerable controversy in the nutritional literature as to the availability of organically bound phosphorus, particularly phytin phosphorus and phytic acid phosphorus. Bruce and Callow ('34) suggest that phytic acid or phytin might be the constituent in cereals which is responsible for their rachitogenic effect. Kreiger et al. ('41) report that phytic acid phosphorus was markedly inferior to inorganic phosphorus for bone calcification in rats. These views are supported by McGinnis et al. ('44) who maintain that inorganic phosphorus is needed for normal bone development in the chick in addition to the organic phosphorus of poultry rations. Singsen and Mitchell ('44) maintain that the phosphorus of plant protein concentrates is available to the chick under certain conditions. Hart and coworkers ('09) indicate that phytin phosphorus is available to the pig. The phosphorus of soybean phosphatides appears to be readily available according to Kreiger et al. ('41).

It has been reported (Morrison, '43) that soybean oil meal contains approximately 0.66% phosphorus. Analyses in this laboratory using the method of Fiske and Subbarow ('25) are in close agreement with this value. The nutritional value of the soybean oil meal phosphorus, however, depends upon its availability to the animal.

Yang and Dju ('39) found that 43% of the total phosphorus of soybeans was present as phytin phosphorus. Earle and Milner ('38) have reported that 75–80% of the total soybean phosphorus is present as phytin and that 10% is present as phospholipid phosphorus. Analyses by the method of McCance and Widdowsen ('35) have shown that 58%

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This study has been supported in part by a grant from the A. E. Staley Manufacturing Company, Decatur, Illinois.

We are indebted to Merck and Co., Rahway, New Jersey, for the synthetic vitamins; to Wilson and Company, Chicago, Illinois, for the 1:20 liver powder; and to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil.

of the total phosphorus of the soybean oil meal used in the studies to be presented here was present in the form of phytin or phytic acid. The remainder existed as inorganic, phospholipid and nucleic acid phosphorus.

Because of the increasingly widespread use of soybean oil meal as a protein supplement in animal feeding, it seemed desirable to determine the availability of its phosphorus.

EXPERIMENTAL

These studies were made with weanling rats averaging 40 gm. in weight and a semi-synthetic basal ration which was low in phosphorus. The basal ration was composed of 14% fibrin [2], 76.6% sucrose, 2% low calcium low phosphorus salts, 4% corn oil, 1% 1:20 liver concentrate to furnish certain vitamins, 0.1% inositol and 0.3% choline chloride. All the animals received a daily vitamin supplement consisting of 30 µg. of riboflavin, 30 µg. of thiamine hydrochloride, 30 µg. of pyridoxine, 100 µg. of calcium pantothenate and 250 µg. of nicotinic acid in a 15% ethyl alcohol solution. This supplement was given by dropper. Halibut liver oil was fed once each week at such a level that it furnished 4,000 U.S.P. units of vitamin A and 70 U.S.P. units of vitamin D. Vitamin K was added to the corn oil at such a level that 100 gm. of ration contained 100 µg. of 2-methyl-1, 4-napthoquinone.

This basal ration furnished 0.028% phosphorus as determined by the method of Fiske and Subbarow ('25) with an Evelyn photoelectric colorimeter. The phosphorus content of all rations was determined in order to provide accurate data on the phosphorus content of the rations at all times throughout the experiment. The calcium content of the ration in all cases was kept as near as possible to 0.6% supplied either as $CaHPO_4$ or $CaCO_3$ or both. Thus the Ca : P ratio while it fluctuated within the limits of 1.4–2.8 : 1 was purposely kept within what we believed to be the limits of a favorable Ca : P ratio since it has been shown that widening of the Ca : P ratio and low vitamin D ingestion results in a decreased utilization of phytic acid phosphorus (Kreiger and Steenbock, '40). Inorganic phosphorus was added as $CaHPO_4$. All major substitutions to the basal ration were made at the expense of the sucrose or fibrin or both.

Rations were stored in the refrigerator to avoid possible development of rancidity. Food and distilled water were fed daily in quanti-

[2] Fibrin ration suggested by D. Klein of Wilson and Company, Chicago, Illinois. Fibrin was used in earlier experiments reported by Jones ('38).

ties to insure ad libitum consumption. The rats were kept in cages of galvanized wire.

At the end of 5 weeks, the animals were sacrificed and the femora removed and used for the determination of bone ash. The bones were extracted with 95% ethyl alcohol for 72 hours, dried, and weighed. They were then ashed at 1600°F. for a period of 2 hours, cooled in a desiccator and the ash weighed.

Experiment I

The first experiment was designed to demonstrate the availability of soybean oil meal phosphorus at two levels of phosphorus feeding. One was used in which soybean oil meal furnished all of the phosphorus except a trace (0.028%) present in the basal ration thus providing a total of 0.256% phosphorus in the ration. This level has been considered by Kreiger et al. ('40) to be adequate for the growing rat. The other level (0.328% phosphorus) furnished slightly more phosphorus than the reported minimum requirement of the rat. Since fibrin was used as the sole source of protein it seemed important to directly compare it with the standard laboratory protein, casein. The protein was kept at a constant level of 14% in all cases. The 1% of 1:20 liver powder furnished less than 0.50% protein and as a result introduced a slight error. Since this was constant in all groups, the effect on the protein level would seem to be negligible especially in view of the estimation that 1% of 1:20 liver powder would contribute at most only 0.004% cystine and 0.008% methionine.

The dietary regimen can be obtained from table 1. Five male and five female weanling rats were used in each lot.

Results

The effect of the basal fibrin-low phosphorus ration, the phosphorus level, and the source of protein upon growth is shown in figure 1. It is seen that little or no growth occurred in the rats fed the basal ration only. Inspection of these data show that the phosphorus (0.228%) of expeller type soybean oil meal was equally as available as 0.228% of inorganic phosphorus for growth since the growth rates of the rats in these two lots were practically identical. It is evident that 0.256% phosphorus was not quite optimum for this period of rapid growth of the rat when daily gains of more than 3 gm. were expected. When the phosphorus level was raised to 0.328%, increased rates of gain in weight were obtained. It is also evident that soybean oil meal furnishes protein which will produce gains in body weight nearly equal to that produced by 14% fibrin when the former is fed on an equivalent

protein basis, i.e., 14% protein or 34% soybean oil meal. Both of these sources of protein were superior to 14% casein in the promotion of growth.

TABLE 1

The availability of soybean oil meal phosphorus (Experiment I).

LOT NO.	NUMBER OF RATS	RATION	PHOSPHORUS IN RATION	CALCIUM IN RATION	BONE ASH AVERAGE
			%	%	%
1	10	Basal	.028	.60	25.6
2	10	Basal + 0.228% inorganic P	.256	.60	48.0
3	10	Basal + 0.3% inorganic P	.328	.60	58.7
4	10	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	.256	.69	54.2
5	10	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P) + 0.072% inorganic P	.328	.69	58.2
6	10	Basal without fibrin + 14% casein (0.11% P) + 0.19% inorganic P	.328	.67	57.8

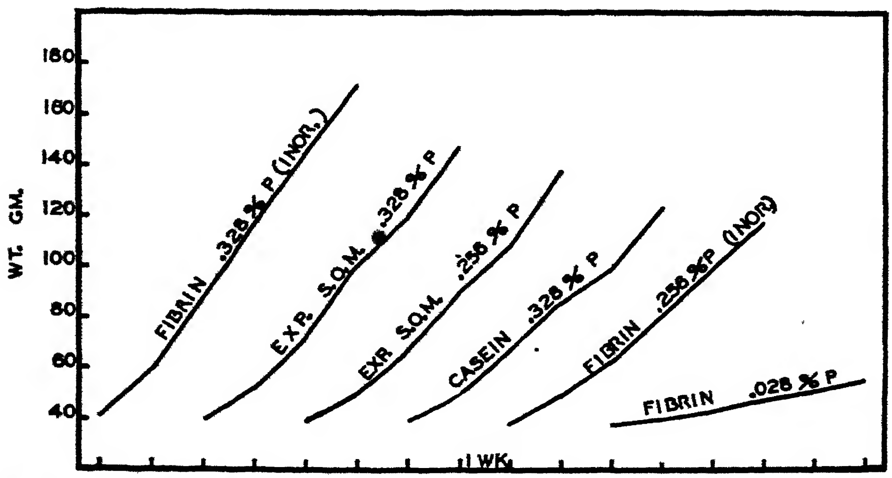


Fig. 1 The effect of expeller soybean oil meal phosphorus and protein upon growth in comparison with inorganic phosphorus and standard laboratory proteins.

It is interesting in this experiment that soybean oil meal (equivalent to 14% protein) caused a slight increase in growth in comparison to fibrin when both rations contained 0.256% phophorus. When the phosphorus level was increased to 0.328%, fibrin increased growth over that of the soybean oil meal fed group. This presents the possibility that phosphorus may influence the biological value of protein under certain conditions.

Table 1 also summarizes data on the availability of soybean oil meal phosphorus, as measured by bone ash. It is evident that the phosphorus of expeller soybean oil meal is as available as inorganic phosphorus furnished as $CaHPO_4$ when they were fed at equivalent levels of 0.256% or 0.328%. When the phosphorus level was increased above 0.256%, a higher bone ash resulted. This gives further evidence to suggest that the optimal phosphorus intake during this period of rapid growth following weaning is greater than 0.256%. Bone ash data indicate that the phosphorus of casein is likewise available.

When the phosphorus content of bone ash was determined by the method of Fiske and Subbarow ('25), it was found that this value was approximately 18% (17.8–18.5%) regardless of the ration ingested.

Experiment II

The second experiment was designed to repeat experiment I, to study the availability of phosphorus in solvent extracted soybean oil meal, to determine the effect of heat on the availability of soybean oil meal phosphorus, to determine if still higher levels of phosphorus would further stimulate growth rate and bone ash deposition, and to determine if casein would support better growth when a higher level of phosphorus was fed.

The dietary regimen is given in table 2. Three male and three female weanling rats were used in each lot except in lot IV where twice this number was used.

Results

The growth rates are indicated by the data given in figure 2. In addition to confirming the results of experiment I, it is seen that the phosphorus requirement of the rat does not exceed 0.328% since further additions of phosphorus failed to support additional growth. The growth obtained with commercial solvent extracted soybean oil meal paralleled that obtained by comparable levels of expeller soybean oil meal. Further additions of phosphorus to the casein ration produced only a slight growth rate response which would seem to indicate that the retarded growth produced by the casein ration was very likely a protein effect.

Data on the availability of soybean oil meal phosphorus as evidenced by bone ash are summarized in table 2. These results are in close agreement with those obtained in experiment I. When inorganic phosphorus was fed at levels of 0.428% and 0.328% phosphorus similar bone ash figures were obtained. This is further evidence which indicates that the

TABLE 2

The availability of soybean oil meal phosphorus (Experiment II).

LOT NO.	NUMBER OF RATS	RATION	PHOSPHORUS IN RATION	CALCIUM IN RATION	BONE ASH AVERAGE
			%	%	%
1	6	Basal	.028	.60	25.4
2	6	Basal + 0.228% inorganic P	.256	.60	50.2
3	6	Basal + 0.3% inorganic P	.328	.60	57.2
4	12	Basal + 0.4% inorganic P	.428	.60	57.2
5	6	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	.256	.69	54.0
6	6	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P) + 0.172% inorganic P	.428	.69	58.5
7	6	Basal without fibrin + 14% casein (0.11% P) + 0.29% inorganic P	.428	.67	57.8
8	6	Basal without fibrin + 34% solvent soybean oil meal (not toasted 14% protein and 0.228% P)	.256	.69	52.4
9	6	Basal without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	.256	.69	53.5
10	6	Basal without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein, 0.228% P) + 0.172% inorganic P	.428	.69	56.7

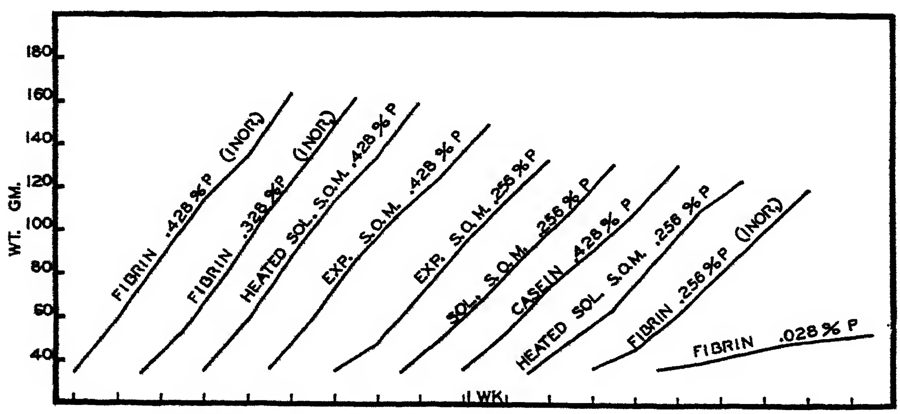


Fig. 2 The effect of expeller and solvent soybean oil meal phosphorus and protein upon growth in comparison with inorganic phosphorus and standard laboratory proteins.

phosphorus requirement of the rat does not exceed 0.328% of the ration. The phosphorus of solvent extracted soybean oil meal appears to be as readily available as that of expeller soybean oil meal. Additional heating of the meal had no effect on the availability of its phosphorus under the conditions of our experiment.

In this experiment as well as in the previous one the protein of soybean oil meal was nearly equal to that of blood fibrin and both of these proteins were distinctly superior to casein when fed at a level of 14% of the ration. These data would indicate that rations using soybean oil meal as the source of protein when properly supplemented with vitamins including choline do not require methionine or cystine in added amounts greater than that supplied by 1% 1:20 liver powder. It is recognized that 1:20 liver powder contains traces of these amino acids. Beach et al. ('43) present data to show that whole liver contains 1.1 to 1.55% cystine and 2.4 to 2.90% methionine. The 1:20 liver powder used in these experiments was a H_2O extract of liver and therefore would not greatly influence the protein levels used. It is estimated that this ration ingredient would add less than 0.004% of cystine and 0.008% methionine, and it is possible that such contamination would be much less than this estimate.

DISCUSSION

The basal ration used in these experiments with 14% blood fibrin as the source of protein makes an excellent low phosphorus ration when low phosphorus salts are employed. It contains on the average only 0.028% phosphorus. The animals on the basal ration grew an average of 0.5 gm. per day over the 5-week experimental period. Their haircoat was rough and diarrhea was common. They were quite inactive and unthrifty. Their bones were very fragile, and averaged only 25.6% ash.

When adequate phosphorus was added to this ration, excellent growth resulted. These rats grew at an average rate of 3.6 gm. per day. Their appearance was excellent, no diarrhea occurred and their bones averaged 58.7% ash. This growth rate was superior to that obtained when casein was fed at an equivalent protein level.

These results indicate clearly that the blood fibrin ration used is an excellent low phosphorus ration, and promotes a very favorable growth rate in young rats when it is adequately supplemented with phosphorus.

Kreiger and coworkers ('40), considered the optimal level of intake of phosphorus for the rat to be 0.257% of the ration. They obtained an average bone ash percentage of 61.5% after 10 to 14 weeks on the ex-

perimental ration. The rats in our experiments were sacrificed after an experimental period of 5 weeks because it was felt that this would permit a more critical comparison of growth rates and of bone ash. Our results show bone ash values of 57 to 59% at the end of 5 weeks and a slightly greater requirement for phosphorus during this period of rapid growth. Since the requirement for phosphorus would be expected to be slightly increased during this interval, the interpretation is that these data are in essential agreement with Kreiger et al. ('40). The phosphorus requirement of the rat immediately following weaning and during the period of rapid growth is greater than 0.256% but less than 0.328% since higher levels of phosphorus feeding had no stimulatory effect. The results obtained in these experiments give definite evidence that the phosphorus in solvent extracted and expeller soybean oil meals is as available as inorganic phosphorus to the rat for bone calcification and growth.

The solvent soybean oil meal and the expeller soybean oil meal used in these experiments contained approximately 0.66% phosphorus of which 58% was in the form of phytin or phytic acid. Kreiger and coworkers ('41) have shown phytic acid phosphorus to be relatively unavailable. These workers (Kreiger and Steenbock, '40) state that it was made less available when the Ca: P ratio was widened and when the vitamin D intake was lowered. It should be pointed out that in the experiments reported in this paper, adequate vitamin D was supplied and the Ca: P ratios were between 1.4:1 and 2.8:1. Furthermore, phytin phosphorus represented a somewhat smaller percentage of the total phosphorus than in the experiments of Kreiger, Bunkfeldt and Steenbock ('40).

It has been suggested by Hart et al. ('09) and by Singsen and Mitchell ('44) that there may be some relationship between the availability of phytin phosphorus and the presence in the ration of an enzyme, phytase, capable of splitting off the phosphate radical from phytin and thereby rendering it available. Phytase has been found in unheated leafy materials and in certain other unaltered natural feedstuffs, but it is destroyed by high temperatures. Heating of solvent soybean oil meal at 98°C. for 48 hours had no effect on the availability of its phosphorus. This suggests that the presence of an enzyme in the soybean oil meal is not required for complete utilization of the phosphorus by the rat. However, it does not rule out the possibility that another constituent in the ration may have carried the "necessary" enzyme. Results of further studies on this subject will be discussed in a subsequent paper.

SUMMARY AND CONCLUSIONS

The 14% blood fibrin ration of Klein has been shown to be an excellent low phosphorus ration (0.028%). Properly supplemented, this ration is capable of sustaining satisfactory to excellent growth rates during the rapid growing period of the young rat. Evidence is presented which indicates that the phosphorus requirement of the young rat during this period lies between 0.256 and 0.328% of the ration.

Soybean oil meals prepared either by the expeller or solvent process, contained 0.66% phosphorus of which 58% was in the form of phytin or phytic acid. The remainder exists as inorganic, phospholipid, and nucleic acid phosphorus.

The phosphorus of soybean oil meal was readily available for growth and bone formation. Additional heat apparently had no measurable effect on the availability of the phosphorus. The phosphorus of casein was likewise readily available. Regardless of the level of phosphorus ingested the bone ash contained approximately 18% phosphorus.

The protein of soybean oil was nearly equal to that of blood fibrin and both of these proteins were superior to casein when they were fed at levels of 14.0%. The data further indicate that soybean oil meal protein when properly supplemented with vitamins including choline does not require cystine and methionine in supplemental amounts greater than that supplied by the trace of these substances present in 1% 1:20 liver powder.

LITERATURE CITED

BEACH, ELIOT F., BERTHA MUNKS AND ABNER ROBINSON 1943 The amino acid composition of animal tissue protein. J. Biol. Chem., vol. 148, p. 431.

BRUCE, H. M., AND R. K. CALLOW 1934 LXXV Cereals and rickets. The role of inositolhexaphosphoric acid. Biochem. J., vol. 28, p. 517.

EARLE, F. R., AND R. T. MILNER 1938 The occurrence of phosphorus in soybeans. Oil and Soap, vol. 15, p. 41.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

HART, E. B., E. V. MCCOLLUM AND J. G. FULLER 1909 The role of inorganic phosphorus in the nutrition of animals. Wisconsin Research Bull. 1.

JONES, JAMES H. 1938 Use of fibrin in synthetic diets. J. Nutrition, vol. 15, p. 269.

KREIGER, C. H., R. BUNKFELDT, C. R. THOMPSON AND H. STEENBOCK 1941 Cereal and rickets. XIII. Phytic acid, yeast nucleic acid, soybean phosphatides, and inorganic salts as sources of phosphorus for bone calcification. J. Nutrition, vol. 21, p. 213.

KREIGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. Availability of phytic acid phosphorus. J. Nutrition, vol. 20, p. 7.

KREIGER, CARL H., AND HARRY STEENBOCK 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutrition, vol. 20, p. 125.

MCCANCE, R. A., AND E. M. WIDDOWSEN 1935 CCCXX. Phytin in human nutrition. Biochem. J. vol. 29, p. 2694.

McGinnis, James, L. C. Norris and G. F. Heuser 1944 Poor utilization of phosphorus in cereals and legumes by chicks for bone development. Poultry Sci., vol. 23, p. 157.

Morrison, F. B. 1943 Feeds and Feeding. Twentieth Edition. The Morrison Publishing Company, Ithaca. N. Y.

Singsen, E. P., and H. H. Mitchell 1944 Soybean meal chick rations need no inorganic phosphorus supplements. Poultry Sci., vol. 23, p. 152.

Yang, E. F., and M. Y. Dju 1939 Total and phytic acid phosphorus in foods. Chinese J. Physiol., vol. 14, p. 473.

ALCOHOL METABOLISM AS RELATED TO THE PRODUCTION OF THIAMINE DEFICIENCY[1]

W. W. WESTERFELD AND E. A. DOISY, JR.
Department of Biological Chemistry, Harvard Medical School, Boston

(Received for publication February 19, 1945)

The occurrence of polyneuritis in chronic alcoholics was recognized 150 years ago (Minot, Strauss and Cobb, '33), and was first believed to be due to a direct "neurotoxic" injury of the nerves by the alcohol. The correct association of this condition with a deficient thiamine intake was established during the past 10 years (Strauss, '35; Jolliffe and Joffe, '35; Jolliffe and Colbert, '36; Jolliffe, Colbert and Joffe, '36; Goodhart and Jolliffe, '38; Jolliffe, '40 b; Wechsler et al., '36; Alexander, '41; and Wortis et al., '42). The inadequate thiamine intake of the chronic alcoholic obviously resulted from the consumption of a large percentage of his total calories in the form of a distilled vitamin-free alcohol. But the results of the clinical studies by Jolliffe and coworkers further indicated that the metabolism of alcohol contributed to the production of the thiamine deficiency because of a requirement for thiamine in the metabolism of the alcohol (Jolliffe, '40 a); in a group of chronic alcoholics the correlation of clinical polyneuritis with the dietary history was significant only when the calories from the alcohol were included in the calculation of the thiamine requirement.

A possible mechanism for the relationship of alcohol metabolism to thiamine was suggested by the theory of intermediary acetoin formation (Westerfeld, Stotz and Berg, '42, '43), in which the acetaldehyde formed by the metabolic oxidation of alcohol would condense with pyruvate under the catalysis of a diphosphothiamine enzyme to form acetoin. It has been shown by in vitro studies that this reaction requires diphosphothiamine (Green et al., '42; Stotz et al., '44), and it is also known that the reaction can take place in vivo (Stozt et al., '44). The rate of alcohol metabolism was not decreased during thiamine deficiency (Berg et al., '44).

The thiamine requirement is known to vary with the composition of the diet; it is increased by a high carbohydrate intake and decreased by

[1] Supported by a grant from the Nutrition Foundation, Inc.

dietary fat (Evans and Lepkovsky, '29, '35; Evans et al., '34; Kemmerer and Steenbock, '33; McHenry, '37; Salmon and Goodman, '37; Arnold and Elvehjem, '39; Ellis and Madsen, '44). This thiamine-sparing action of fat is based primarily upon the longer dietary period required to produce deficiency symptoms on a high-fat diet. A similar thiamine-sparing action of alcohol has recently been demonstrated by Lowry et al. ('42) by paired feeding experiments in rats.

The experiments herein described were carried out because of the apparent contradiction in the results obtained by Jolliffe and coworkers and Lowry and associates, and because of the bearing this problem has on the role of acetoin in alcohol metabolism. It was found that the onset of opisthotonus and death in pigeons on a thiamine-deficient diet was delayed when alcohol was substituted isocalorically for either fat or carbohydrate in the diets. It is obvious therefore that there is no increased requirement for thiamine resulting from the metabolism of alcohol — at least, when alcohol is not the major source of the calories. The results do not furnish any support for the formation of acetoin in the intermediary metabolism of alcohol, nor do they eliminate this possibility.

TABLE 1

Composition of the diets.

DIET	I		II		III		IV	
	gm.	*Cal.*	*gm.*	*Cal.*	*gm.*	*Cal.*	*gm.*	*Cal.*
Casein (S.M.A. vitamin-free)	23	92	23	92	23	92	23	92
Autoclaved yeast [1]	15	50	15	50	15	50	15	50
Cod liver oil	3	27	3	27	3	27	3	27
Salt mixture (Phillips and Hart)	4		4	.	4	..	4	
Corn oil (Mazola)	4	36	4	36	20	180	13	117
Sucrose	51	204	34	136	15	60	15	60
Alcohol	.	..	9	64	.	..	9	64
Total [2]	100	409	92	405	80	409	82	410

[1] Anheuser-Busch Strain K brewers' yeast autoclaved at 15 lb. for 6 hours. Composition = 45–50% protein: 3.5–4.0 Cal./gm.
[2] Divided into four daily feedings.

EXPERIMENTAL

Four isocaloric diets were used in these experiments, and their compositions are given in table 1. Diet I was high in carbohydrate, part of which was replaced isocalorically by alcohol in diet II and by fat in

diet III. Diet IV was identical with III except that part of the fat was replaced isocalorically by alcohol. The diets were further supplemented so that each pigeon received an additional 50 mg. choline chloride daily as well as 10 mg. α-tocopherol and 0.1 mg. 2-methyl, 1:4-naphthoquinone twice weekly. Diets II and IV were prepared without addition of the alcohol, and the latter was added immediately prior to feeding. A weighed quantity of each diet, equivalent to approximately 100 cal., was force-fed daily by mixing with water and delivering it into the crop (Swank and Bessey, '41). The birds on all four diets were directly comparable, since each received daily the same number of calories and the same amounts of all the dietary constituents not under investigation.

Series I

Four dozen White King pigeons were divided into four comparable groups on the basis of body weights and each group was fed one of the experimental diets until the appearance of opisthotonus and death. The dietary period preceding the onset of thiamine deficiency was the principal point of study, and these results, together with body-weight changes, are recorded in table 2.

The average dietary period preceding death on diets I to IV was 19, 25, 27 and 42 days, respectively. Three of the pigeons on diet IV were sacrificed on the sixty-ninth day, so that the average of this group would have been still longer if the experiment had not been terminated. Comparison of the results obtained with diet II vs. diet I and on diet IV vs. diet III showed that the substitution of alcohol for either carbohydrate or fat delayed the onset of acute thiamine deficiency. The well known thiamine-sparing action of fat was again confirmed (diet III vs. diet I). About the same thiamine-sparing action was exerted by 16 gm. fat (144 Cal.) or 9 gm. alcohol (64 Cal.) (diets II and III vs. diet I).

As a group, the pigeons were much less consistent in their response to the diets containing alcohol. The dietary period preceding death varied from 12 to 23, 17 to 43, 20 to 34, and 20 to 69 days on diets I to IV, respectively, for a corresponding spread of 11, 26, 14, and 49 days. The inclusion of alcohol in both diets II and IV increased the individual variation as shown by the greater spread of the results. While the differences in the median values of 19.5, 24, 27, and 34 days for the four groups clearly indicate that each group as a whole responded to the diets in the manner indicated by the previously cited averages, it is also clear that the average obtained with diet IV is weighted by a few birds that survived an unusually long time.

TABLE 2

Weight changes, onset of deficiency symptoms and survival in series I.

DIET NO. 1					DIET NO. 2				
Weight in gm.			Days on experiment		Weight in gm.			Days on experiment	
Start	Onset of symptoms	Death	Onset of symptoms [1]	Death	Start	Onset of symptoms	Death	Onset of symptoms [1]	Death
332	311	261	15 [2]	16	369	331	292	17 [2]	18
412	386	339	14	16	407	. .	262	.	24
412	338	305	18	19	419	349	355	23	24
433	410	385	17 [2]	17	432	.	326		21
438	333	319	22	23	439	314	263	23 [2]	27
456	409	403	19	21	450	. .	325		17
462	321	289	19	20	463	336		27–29	
481	. . .	402	11 [2]	12		313	296	42	43
485	. .	328	. .	20	488	. .	451	30 [2]	31
511	. .	371	. .	22	480	413	369	17 [2]	18
519	. .	379	.	17	500	.	280		26
640	466	466	21 [2]	22	530	381	368	32	33
					574		332		21
465	Average	354		19	463	Average	327		25
DIET NO. 3					DIET NO. 4				
370	312	293	27	29	390	420	356	34	36
404	. . .	388	.	23	401	. . .	436	.	69 [3]
420	433	433	18	20	420	414	414	23	25
431	. . .	410		34	428	321	366	37–38	69 [3]
441	428	398	26	27	441	438	423	29	32
448	397	368	23	24	446	. .	301		20
465	411	380	23	24	467	358	344	27 [2]	28
473	359	353	27	28	471	. . .	459	. .	23
488	453	423	23	25	490	416	406	25	28
491	367	350	31	33	491	. .	382	.	47
549	463	453	26	27	562	425		32–38	
627	519	515	25	27		407	407	69	69 [3]
					575		432	57 [2]	58
467	Average	397		27	465	Average	394		42

[1] Classical opisthotonus unless marked [2]. [2] Indicates pronounced leg weakness or paralysis without head retraction. [3] Sacrificed.

The development of thiamine deficiency symptoms also differed in the various groups. The percentage of each group that passed through the classical head retraction and tumbling syndrome varied from about 50% on diets I and IV, 25% on diet II, and 80% on diet III. Marked deficiency symptoms without opisthotonus occurred in some of the other birds but about $\frac{1}{2}$ of the birds on diet II, $\frac{1}{3}$ on diet IV, $\frac{1}{4}$ on diet I, and $\frac{1}{8}$ of the birds on diet III died without marked symptoms heralding the event. Thus both diets containing alcohol tended to prevent the ante-mortem appearance of acute thiamine deficiency symptoms. If these birds died suddenly from some effect of the alcohol rather than from the thiamine deficiency, then the thiamine-sparing action of the alcohol would be greater than indicated by the results actually obtained.

The interval between the appearance of opisthotonus and death on diets I and II was usually no more than 1 day; on diets III and IV, it averaged 1.5 and 2.5 days, respectively. Two of the birds on diet IV and one on diet II developed classical opisthotonus that remained for 1 to 6 days and then spontaneously disappeared; it reappeared again in two of the birds after intervals of 13 to 31 days. This phenomenon has never been observed in any of the birds which were force-fed with non-alcoholic diets. Following the spontaneous disappearance of opisthotonus, these pigeons exhibited a very marked leg weakness that persisted until subsequent death. Thus the inclusion of alcohol in the diets allowed a few of the birds to recover from the marked deficiency symptoms, that invariably led to rapid death on other diets, and continue to exist in a state of marked but sub-acute deficiency.

Comparison of the weight loss in the various groups shows rather conclusively that vomiting and any resultant inanition that might have occurred during the latter stages of the deficiency was not an important factor invalidating the results. The weight loss of the birds on diets III and IV was about the same in spite of the longer survival of group IV, and both groups lost less weight than those on diet I. Only on diet II was the longer survival associated with a greater weight loss, and this effect could be associated with the longer survival per se or the metabolic effect of alcohol as well as with any inanition resulting from vomiting.

Series II

In a repetition of these studies, the experiments were designed to eliminate some of the variables present in the first series. All of the birds were first brought into opisthotonus by the daily force-feeding of diet I. When opisthotonus appeared, each bird was given 25 µg.

thiamine intramuscularly and placed permanently on one of the four diets previously described. For 2 weeks thereafter, each bird received daily 10 μg. thiamine intramuscularly to control vomiting, and at the end of this period the thiamine administration was stopped. The time elapsed from the withdrawal of thiamine to the reappearance of opisthotonus was the principal criterion of the relative thiamine requirement of the various diets. By this procedure, the various diets were "titrated" against the administered thiamine, since the end-point of the experiment for each bird was a return to the same state of opisthotonus with which it was started on the diet. Only those pigeons that developed a classical opisthotonus from diet I were used in the experiment, and the birds that did not recover properly after the first thiamine injection were discarded.

The results, which are recorded in table 3, are essentially the same as those obtained in the previous series. The last five birds listed in diets III and IV were run as a separate group from the others. The thiamine-sparing action of alcohol and fat was shown by the survival periods of 3, 10, 9, and 16 days for diets I to IV, respectively. From the rapidity of onset of opisthotonus, it would appear that 10 μg. thiamine daily approximated the minimum requirement for existence on diet I for the 2-week period studied. The preliminary depletion of the thiamine stores in the body did not eliminate the variation in response between birds. There was some correlation within each group of the rapidity of onset of opisthotonus on diet I and the later reappearance of opisthotonus on the other diets. A few birds that were temporarily relieved of opisthotonus by the administration of thiamine while still on diet I were less resistent to the subsequent development of thiamine deficiency.

Vomiting was negligible during the thiamine administration but increased after its cessation and may have been somewhat greater in group II. In general, intense vomiting indicated the approach of the more deficient symptoms of leg weakness and opisthotonus. The preliminary development of opisthotonus on diet I was accompanied by a loss of 16 to 19% of the original body weight. Only the birds on diet III regained a fair proportion of this weight during the administration of 10 μg. thiamine daily, and only group II lost an additional portion of its weight prior to the reappearance of opisthotonus.

Although the experimental results were completed at the second appearance of opisthotonus, some of the birds were injected daily with an excess of thiamine and continued on the same dietary regime for an additional month. Restoration of weight was accomplished most readily

on diet III, somewhat less readily on diets I and IV, and least readily on diet II; the increase in weight was retarded by the alcohol. All four diets, when supplemented with thiamine, were adequate for maintenance of these pigeons, and the deficiency observed was justifiably attributed to thiamine.

TABLE 3

Weight changes and dietary periods preceding opisthotonus in series II.

DIET NO.	ORIGINAL WEIGHT	OPISTHOTONUS FROM DIET I		AFTER 2 WEEKS ON DIETS I–IV WITH THIAMINE	OPISTHOTONUS AFTER THIAMINE WITHDRAWAL	
		Dietary period	Weight	Weight	Dietary period	Weight
	gm.	*days*	*gm.*	*gm.*	*days*	*gm.*
I	339	15	317	324	3 [2]	321
	484	18	341	372	1	358
	578	19	485	510	6	487
	493	25 [1]	443	435	2 [2]	426
Av.	474	19	397	410	3	398
II	362	17	322	313	6	261
	642	19	483	489	12	412
	521	21 [1]	449	433	6	374
	563	22	454	497	16	431
Av.	522	20	427	433	10	370
III	437	15	364	410	11	353
	479	17	420	471	12	390
	451	17	351	412	10	367
	431	19	329	418	13	370
	516	13	435	400	8	381
	459	14	368	400	6	362
	373	14	305	375	6	346
	394	15	328	375	12	387
	477	16	384	420	7	400
Av.	446	16	365	409	9	373
IV	515	16	397	427	10	404
	468	17 [1]	402	416	7	387
	515	19	390	394	15	348
	351	13	303	363	15	376
	490	13	389	402	10	380
	576	14	487	463	14	448
	423	15	342	370	17 [2]	375
	613	17	479	452	39	450
Av.	494	16	399	411	16	396

[1] Previous opisthotonus on the 13th day temporarily relieved by thiamine.

[2] Death without preceding opisthotonus.

thiamine intramuscularly and placed permanently on one of the four diets previously described. For 2 weeks thereafter, each bird received daily 10 μg. thiamine intramuscularly to control vomiting, and at the end of this period the thiamine administration was stopped. The time elapsed from the withdrawal of thiamine to the reappearance of opisthotonus was the principal criterion of the relative thiamine requirement of the various diets. By this procedure, the various diets were "titrated" against the administered thiamine, since the end-point of the experiment for each bird was a return to the same state of opisthotonus with which it was started on the diet. Only those pigeons that developed a classical opisthotonus from diet I were used in the experiment, and the birds that did not recover properly after the first thiamine injection were discarded.

The results, which are recorded in table 3, are essentially the same as those obtained in the previous series. The last five birds listed in diets III and IV were run as a separate group from the others. The thiamine-sparing action of alcohol and fat was shown by the survival periods of 3, 10, 9, and 16 days for diets I to IV, respectively. From the rapidity of onset of opisthotonus, it would appear that 10 μg. thiamine daily approximated the minimum requirement for existence on diet I for the 2-week period studied. The preliminary depletion of the thiamine stores in the body did not eliminate the variation in response between birds. There was some correlation within each group of the rapidity of onset of opisthotonus on diet I and the later reappearance of opisthotonus on the other diets. A few birds that were temporarily relieved of opisthotonus by the administration of thiamine while still on diet I were less resistent to the subsequent development of thiamine deficiency.

Vomiting was negligible during the thiamine administration but increased after its cessation and may have been somewhat greater in group II. In general, intense vomiting indicated the approach of the more deficient symptoms of leg weakness and opisthotonus. The preliminary development of opisthotonus on diet I was accompanied by a loss of 16 to 19% of the original body weight. Only the birds on diet III regained a fair proportion of this weight during the administration of 10 μg. thiamine daily, and only group II lost an additional portion of its weight prior to the reappearance of opisthotonus.

Although the experimental results were completed at the second appearance of opisthotonus, some of the birds were injected daily with an excess of thiamine and continued on the same dietary regime for an additional month. Restoration of weight was accomplished most readily

on diet III, somewhat less readily on diets I and IV, and least readily on diet II; the increase in weight was retarded by the alcohol. All four diets, when supplemented with thiamine, were adequate for maintenance of these pigeons, and the deficiency observed was justifiably attributed to thiamine.

TABLE 3

Weight changes and dietary periods preceding opisthotonus in series II.

DIET NO.	ORIGINAL WEIGHT	OPISTHOTONUS FROM DIET I		AFTER 2 WEEKS ON DIETS I–IV WITH THIAMINE	OPISTHOTONUS AFTER THIAMINE WITHDRAWAL	
		Dietary period	Weight	Weight	Dietary period	Weight
	gm.	*days*	*gm.*	*gm.*	*days*	*gm.*
I	339	15	317	324	3 [2]	321
	484	18	341	372	1	358
	578	19	485	510	6	487
	493	25 [1]	443	435	2 [2]	426
Av.	474	19	397	410	3	398
II	362	17	322	313	6	261
	642	19	483	489	12	412
	521	21 [1]	449	433	6	374
	563	22	454	497	16	431
Av.	522	20	427	433	10	370
III	437	15	364	410	11	353
	479	17	420	471	12	390
	451	17	351	412	10	367
	431	19	329	418	13	370
	516	13	435	400	8	381
	459	14	368	400	6	362
	373	14	305	375	6	346
	394	15	328	375	12	387
	477	16	384	420	7	400
Av.	446	16	365	409	9	373
IV	515	16	397	427	10	404
	468	17 [1]	402	416	7	387
	515	19	390	394	15	348
	351	13	303	363	15	376
	490	13	389	402	10	380
	576	14	487	463	14	448
	423	15	342	370	17 [2]	375
	613	17	479	452	39	450
Av.	494	16	399	411	16	396

[1] Previous opisthotonus on the 13th day temporarily relieved by thiamine.
[2] Death without preceding opisthotonus.

DISCUSSION

The results show that the isocaloric substitution of alcohol for dietary fat or carbohydrate delays the onset of acute thiamine deficiency symptoms. If this thiamine-sparing action represents a decreased utilization of thiamine in the metabolism of the foodstuffs ingested, then the simplest explanation of the results obtained is that alcohol requires less thiamine for its metabolism than carbohydrate. While the thiamine requirement for alcohol could theoretically be zero from these experiments, the sparing action observed when alcohol was substituted for fat could be interpreted as indicating a thiamine requirement for the metabolism of fat intermediate between those for carbohydrate and alcohol.

An equally possible explanation is that the metabolism of alcohol and carbohydrate together requires less thiamine than the metabolism of carbohydrate alone. Thus the thiamine requirement of diets I and III would be due primarily to their respective carbohydrate contents, and the sparing action exerted by diets II and IV would be due to the metabolism of the alcohol and carbohydrate together. This effect would occur if (1) the metabolism of carbohydrate in the presence of alcohol followed a different pathway (requiring less thiamine) from that taken during the metabolism of carbohydrate alone, or (2) the metabolism of alcohol spared the oxidation of carbohydrate.

An attempt was made to study the thiamine requirement when alcohol was substituted in a carbohydrate-free diet, so that any effect of the alcohol could not be attributed to the combination of alcohol and carbohydrate. Diet V contained 11 gm. casein, 15 gm. autoclaved yeast, 3 gm. cod liver oil, 4 gm. salt mixture, and 32 gm. corn oil, and in diet VI, 9 gm. alcohol were substituted for 7 gm. of the corn oil. These diets were supplemented and force-fed to pigeons as in series I, but the experiment was terminated after 40 days because $\frac{3}{4}$ of the birds on diet VI and $\frac{1}{4}$ on diet V had died without showing the characteristic thiamine deficiency symptoms. Deaths started on the sixth and tenth days of diets VI and V, and were scattered throughout the remainder of the dietary period. Most of the birds that died had a greatly enlarged liver (8 to 35 gm. as compared with normals of 5 to 11 gm.) with an inversely correlated fat content (1.6 to 5% of the wet weight as compared with normal values of 3.5 to 5%. Whatever the cause of death, no conclusions could be reached regarding the thiamine requirements of the two diets.

In general, these results confirm and extend those reported for rats by Lowry et al. ('42), and are contradictory to the assumption that

alcohol metabolism increases the thiamine requirement. It is difficult to make the obvious conclusion from Jolliffe's work that the metabolism of alcohol requires a small but definite amount of thiamine, since the thiamine requirement for the diets containing alcohol would probably be less than that calculated by Cowgill's formula ('34). It is possible that the thiamine equivalent of alcohol is increased when the latter contributes a very large percentage of the total daily calories.

SUMMARY

The isocaloric substitution of alcohol for fat or carbohydrate in a thiamine-deficient diet delays the onset of opisthotonus and death in pigeons.

LITERATURE CITED

ALEXANDER, L. 1941 Neuropathological findings in the brain and spinal cord of chronic alcoholic patients. Quart. J. Studies Alc., vol. 2, p. 260.

ARNOLD, A., AND C. A. ELVEHJEM 1939 Influence of the composition of the diet on the thiamine requirement of dogs. Am. J. Physiol., vol. 126, p. 289.

BERG, R. L., E. STOTZ AND W. W. WESTERFELD 1944 Alcohol metabolism in thiamine deficiency. J. Biol. Chem., vol. 152, p. 51.

COWGILL, G. R. 1934 The Vitamin B Requirement of Man. Yale University Press, New Haven.

ELLIS, N. R., AND L. L. MADSEN 1944 The thiamine requirement of pigs as related to the fat content of the diet. J. Nutrition, vol. 27, p. 253.

EVANS, H. M., AND S. LEPKOVSKY 1929 Sparing action of fat on the anti-neuritic vitamin B. J. Biol. Chem., vol. 83, p. 269.

——— 1935 The sparing action of fat on vitamin B. VIII. On the loss of vitamin B from the rat's tissues. J. Biol. Chem., vol. 108, p. 439.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 The sparing action of fat on vitamin B. VII. The effectiveness of various natural fats in sparing vitamin B. J. Biol. Chem., vol. 107, p. 439.

GOODHART, R., AND N. JOLLIFFE 1938 Effects of vitamin B (B_1) therapy on the polyneuritis of alcohol addicts. J. Am. Med. Assn., vol. 110, p. 414.

GREEN, D. E., W. W. WESTERFELD, B. VENNESLAND AND W. E. KNOX 1942 Carboxylases of animal tissues. J. Biol. Chem., vol. 145, p. 69.

JOLLIFFE, N. 1940 a The influence of alcohol on the adequacy of the B vitamins in the American diet. Quart. J. Studies Alc., vol. 1, p. 74.

——— 1940 b Vitamin deficiencies and liver cirrhosis in alcoholism. Quart. J. Studies Alc., vol. 1, p. 517.

JOLLIFFE, N., AND C. N. COLBERT 1936 The etiology of polyneuritis in the alcohol addict. J. Am. Med. Assn., vol. 107, p. 642.

JOLLIFFE, N., C. N. COLBERT AND P. M. JOFFE 1936 Observations on the etiologic relationship of vitamin B (B_1) to polyneuritis in the alcohol addict. Am. J. Med. Sci., vol. 191, p. 515.

JOLLIFFE, N., AND P. M. JOFFE 1935 Relation of vitamin B (B_1) intake to neurological changes in the alcohol addict. Proc. Soc. Exp. Biol. Med., vol. 32, p. 1161.

KEMMERER, A. R., AND H. STEENBOCK 1933 A study of the sparing action of fats on the vitamin B content of animal tissues. J. Biol. Chem., vol. 103, p. 353.

LOWRY, J. V., W. H. SEBRELL, F. S. DAFT AND L. L. ASHBURN 1942 Polyneuropathy in thiamine deficient rats delayed by alcohol or whiskey. J. Nutrition, vol. 24, p. 73.

McHENRY, E. W. 1937 An effect of choline on the vitamin B_1-sparing action of fats. Biochem. J., vol. 31, p. 1616.
MINOT, G. R., M. B. STRAUSS AND S. COBB 1933 "Alcoholic" polyneuritis; dietary deficiency as a factor in its production. New Eng. J. Med., vol. 208, p. 1244.
SALMON, W. D., AND J. G. GOODMAN 1937 Alleviation of vitamin B deficiency in the rat by certain natural fats and synthetic esters. J. Nutrition, vol. 13, p. 477.
STOTZ, E., W. W. WESTERFELD AND R. L. BERG 1944 The metabolism of acetaldehydate with acetoin formation. J. Biol. Chem., vol. 152, p. 41.
STRAUSS, M. B. 1935 The etiology of "alcoholic" polyneuritis. Am. J. Med. Sci., vol. 189, p. 378.
SWANK, R. L., AND O. A. BESSEY 1941 III. Avian thiamine deficiency. Characteristic symptoms and their pathogenesis. J. Nutrition, vol. 22, p. 77.
WECHSLER, I. S., G. JERVIS AND H. D. POTTS 1936 Experimental study of alcoholism and vitamin B deficiency in monkeys. Bull. Neurol. Inst., N. Y., vol. 5, p. 453.
WESTERFELD, W. W., E. STOTZ AND R. L. BERG 1942 The role of pyruvate in the metabolism of ethyl alcohol. J. Biol. Chem., vol. 144, p. 657.
——— 1943 The coupled oxidation-reduction of alcohol and pyruvate in vivo. J. Biol. Chem., vol. 149, p. 237.
WORTIS, H., E. BUEDING AND N. JOLLIFFE 1942 Pyruvic acid studies in the peripheral neuropathy of alcohol adicts. New Eng. J. Med., vol. 226, p. 376.

A STUDY OF HEMOGLOBIN FORMATION FOLLOWING THE ADMINISTRATION OF CERTAIN AMINO ACIDS TO RATS FED A DIET LOW IN PROTEIN[1]

ALINE UNDERHILL ORTEN AND JAMES M. ORTEN
Department of Physiological Chemistry, Wayne University College of Medicine, Detroit

(Received for publication April 30, 1945)

The fact that an adequate intake of dietary protein is essential for normal hematopoiesis in the rat has been demonstrated in previous studies in this laboratory. A mild chronic anemia resulted from the administration of a diet low in protein but adequate in all other respects and the anemia was alleviated by increasing the protein allowance without altering the intake of calories, iron, vitamins, or other dietary constituents (Orten and Orten, '43a). On the other hand, administration of iron alone had no consistent beneficial effect. Further evidence of the importance of dietary protein for hemoglobin formation has been obtained by other investigators in studies on hemorrhagic anemia in dogs (Hahn and Whipple, '39; Sturgis and Farrar, '35), the anemia of pregnancy in man (Bethell, '36), and nutritional anemia in infants (Bass, '44).

The question of the identity of the amino acids involved in the hematopoietic effect of protein has received some attention. It appears possible that the action may reside either in some "key" amino acid or in some combination of a few amino acids. Some support to this possibility is found in the claims that tryptophane and histidine (Fontes and Thivolle, '30) and cystine, proline, and certain other individual amino acids (Whipple and Robscheit-Robbins, '40) increase the rate of hemoglobin regeneration in the hemorrhagic anemia of dogs. Furthermore, anemia may be produced in rats by the feeding of a diet deficient in lysine (Hogan, Powell and Guerrant, '41; Harris, Neuberger and Sanger, '43) or in tryptophane (Albanese et al., '43). It may also be pointed out that the hemoglobin molecule is characterized by a relatively high content of histidine anl lysine (Block and Bolling, '43) and hence that one or both of these individual amino acids might be expected to exert

[1] Aided by a grant from the Committee on Research, American Association for the Advancement of Science. Preliminary reports were made before the American Society of Biological Chemists at Toronto, 1939 and the American Institute of Nutrition in New Orleans, 1940.

a demonstrable hematopoietic effect, particularly if the intake of dietary protein were insufficient.

On the other hand, it is perhaps more logical to expect that a mixture of amino acids in as yet unknown proportions is essential for normal hematopoiesis. If this is the case no increase in hemoglobin formation should follow the administration of any individual amino acid, providing, of course, that the amino acid in question was not the only one lacking in the diet.

The present study was designed to investigate the foregoing possibilities by administering singly the "essential" and certain non-essential amino acids to rats having a chronic anemia due to a low-protein intake.

EXPERIMENTAL

Twenty-one-day-old female rats of the Connecticut Agricultural Experiment Station strain, weighing from 40 to 50 gm., were used. They were placed in individual cages and fed the following low-protein diet: lactalbumin, 3.5%; dextrin, 55.5%; sucrose, 10%; hydrogenated cottonseed oil, 27%; and Wesson salt mixture, 4%. Control animals were given the same basal ration with the exception that it contained 18% lactalbumin, replacing an equivalent amount of dextrin. All animals were given 200 mg. "Ryzamin B"[2] and 100 mg. liver extract[3] daily and 2 drops of a cod liver oil concentrate twice weekly.

After the experimental animals had been fed the low-protein diet for 100 days and had developed the retarded somatic growth and characteristic anemia previously described (Orten and Orten, '43a) supplementation with various individual amino acids, or with lactalbumin in one group, was begun. A daily amount of each amino acid equivalent to that present in the quantity of lactalbumin consumed daily by normal control rats, was administered. These amounts, given in table 1, together with the optical forms used, were calculated from accepted analyses of the amino acid composition of lactalbumin (Schmidt, '38) except for threonine which was administered arbitrarily at a level of 50 mg. per day. In the case of isoleucine, the value obtained from analytical data was doubled, since the d-l form was used. The amino acids were incorporated directly in the basal low-protein diet which was fed in the same average daily amount (found to be 4.0 gm.) as had been consumed by the low-protein animals during the final 2 weeks of the preliminary period. The purpose of the restriction of total food intake

[2] Burroughs Wellcome and Company, Tuckahoe, New York.

[3] Eli Lilly and Company, no. 343. Appreciation is expressed to Dr. C. P. Rhodehamel for a generous supply of this material.

was to make certain that any changes in hematopoiesis following supplementation would be due to the added amino acid itself rather than to an increased food intake. Amino acid supplementation was continued for as long as 12 weeks in groups in which a sufficient number of rats survived to make this possible.

Hemoglobin determinations were made weekly by a photoelectric acid hematin method on blood obtained from a tail vein.

RESULTS

The averaged results of hemoglobin determinations, together with minimum and maximum individual values, are given in table 1.

It is evident that the normal control rats fed the adequate protein diet maintained normal hemoglobin values during the entire experimental period whereas the animals continued on the low-protein diet remained in a chronic state of mild anemia. The group of animals given a restricted amount (4.0 gm. daily) of the basal low-protein diet supplemented by lactalbumin in an amount consumed by normal control rats (replacing dextrin iso-calorically), showed a prompt increase in the concentration of hemoglobin in the blood to a normal level, as previously reported (Orten and Orten, '43a). Normal hemoglobin values were maintained throughout the 12-week period of observation. This finding also demonstrated that no apparent irreversible pathological change had taken place in the protein-deficient rats during the 100-day pre-experimental period and that a return to normal, hematologically as well as in gross respects, was possible provided supplementation was adequate.

There was some variation in the response to the administration of the different individual amino acids but in no case was a sustained favorable effect observed either on the hemoglobin content of the blood, on body weight, or on the gross appearance of the animal. Glycine, used primarily as a control "non-essential" amino acid, produced a transient rise in hemoglobin of doubtful significance. This was followed by a steady decrease in hemoglobin values during the remainder of the period of observation. Similar results were obtained with the other "non-essential" amino acids studied, namely, cystine, glutamic acid, proline, and tyrosine, and with the 10 "essential" amino acids. Transient rises in the hemoglobin level were observed in some of the rats but the increases were neither consistent nor sustained. Of special interest are the failure of histidine and tryptophane, alleged to have special hematopoietic properties by other investigators (Fontes and Thivolle, '30), to produce a significant rise in hemoglobin.

TABLE 1

Hemoglobin content of the blood of control rats and of rats fed a low-protein diet supplemented by various amino acids.

DIET	NO. OF RATS	DAILY SUPPLEMENT MG.	HEMOGLOBIN — GM. PER 100 ML.[1]				
			Initial	3 weeks	6 weeks	9 weeks	12 weeks
18% lact-albumin	12	...	16.5 (15.7–18.0)	16.6 (15.6–19.6)	16.3 (15.5–16.6)	16.7 (16.0–18.0)	16.1 (15.8–16.8)
3.5% lact-albumin	10	.	12.2 (10.3–13.6)	12.1 (10.9–14.2)	12.1 (10.2–14.2)	11.7 (10.5–13.3)	11.3 (10.2–11.7)
3.5% + lact-albumin	12	1,660	11.5 (9.6–13.5)	14.9 (14.1–16.0)	16.1 (15.4–17.4)	16.1 (14.8–17.2)	15.4 (13.5–16.3)
3.5% + glycine	6	50	12.4 (9.7–13.6)	12.9 (9.0–14.8)	11.8 (4.0–15.2)	10.2	10.2 (........)
3.5% + l-cystine	4	77	12.3 (12.0–12.5)	12.2 (10.9–12.7)	12.2 (10.6–13.9)	11.5 (9.9–12.7)	12.2 (10.4–13.7)
3.5% + l-glutamic acid	6	232	11.6 (9.3–13.3)	11.1 (6.0–13.2)	11.0 (9.2–12.8)	9.4 (7.3–11.5)	....
3.5% + l-proline	4	68	12.1 (11.3–13.2)	12.1 (10.2–14.0)	13.4 (12.2–14.6)	12.9 (12.4–13.5)	.....
3.5% + l-tyrosine	4	35	12.3 (11.1–13.5)	13.3 (12.2–14.4)	11.9 (9.5–14.2)	10.9 (7.6–14.2)	....
3.5% + l-arginine	6	63	11.7 (10.2–13.3)	12.7 (9.7–15.0)	....	13.2 (11.5–13.8)	....
3.5% + l-histidine	6	50	12.7 (10.0–14.0)	12.8 (10.7–14.0)	13.0 (10.5–14.2)	12.0 (... .)	.....
3.5% + dl-leucine	4	230	13.3 (13.0–13.6)	13.8 (12.8–15.1)	13.1 (12.2–13.6)	....	....
3.5% + dl-isoleucine	4	50	12.2 (11.5–13.0)	12.6 (11.8–13.2)	13.6 (12.8–14.5)	.....	....
3.5% + dl-lysine	5	178	12.0 (10.8–13.2)	11.6 (11.0–12.2)	13.5 (13.3–13.7)	12.6 (11.9–13.3)	....
3.5% + dl-methionine	4	47	11.4 (10.3–12.8)	11.5 (10.2–13.1)	11.5 (10.7–12.4)	11.6 (9.8–13.1)	11.3 (9.6–13.0)
3.5% + dl-phenylalanine	4	23	12.6 (11.9–13.2)	13.5 (13.1–13.7)	13.1 (12.6–13.7)	12.6 (12.2–13.0)	....
3.5% + l-tryptophane	6	45	12.4 (10.3–14.0)	13.2 (11.9–14.4)	13.3 (12.7–14.2)	12.5 (12.3–12.8)	11.1 (10.8–11.4)
3.5% + dl-threonine	4	50	12.3 (12.2–12.5)	12.9 (12.2–13.7)	12.8 (12.3–13.2)	.....	...
3.5% + dl-valine	8	60	11.5 (8.5–13.7)	11.8 (8.0–14.6)	12.1 (8.2–15.4)	13.1 (12.9–13.5)	......

[1] Values given are group averages. Minimum and maximum individual values are given in

DISCUSSION

The foregoing results indicate that, under the conditions employed, none of the individual amino acids studied exerts any unique hematopoietic effect and therefore cannot be regarded as a "key" amino acid in hemoglobin synthesis.

These results obviously do not support the claims for a special hematopoietic value of such individual amino acids as tryptophane and histidine (Fontes and Thivolle, '30) or certain other individual amino acids (Whipple and Robscheit-Robbins, '40). However, in these investigations a different experimental approach to the problem and a different species of animal were used which may possibly account for the apparent discrepancy between their results and ours. Perhaps a more probable explanation is that other necessary amino acids for hemoglobin synthesis were supplied by the animal's own tissue protein, a reserve which could have been drawn upon in either of the two studies in question. The results obtained in the present study, however, are not at variance with those of Hogan, Powell, and Guerrant ('41) showing that the amino acid lysine will cure the anemia produced in rats by the feeding of deaminized casein. In this work there was apparently a deficiency of only one amino acid, rather than a multiple deficiency, and it is logical to find that the supplying of the missing amino acid was followed by a hematological response. The same is true of the studies (Harris et al., '43; Albanese et al., '43) demonstrating that tryptophane administration will correct the anemia observed in rats fed a diet deficient in this one amino acid.

The results of the present investigation suggest that a combination of amino acids in as yet undetermined proportions is needed for the synthesis of the hemoglobin molecule. This view is supported by the recent observation in this laboratory that beef blood protein is strikingly inferior to casein for growth and hemoglobin formation in the rat (Orten and Orten, '43b, '44) and that the amino acids present in beef and human globin, which are deficient in iso-leucine, do not support normal hematopoiesis unless iso-leucine is added to the diet (Orten, Orten and Bourque, '45). Further, these observations indicate that the protein supplying the proportions of amino acids best suited to the general synthesis of body tissue proteins, as shown by a superior rate of growth, also supplies a combination of amino acids best suited for the synthesis of hemoglobin.

SUMMARY

The effects of the administration of various individual amino acids on the hemoglobin content of the blood of rats made anemic by the ingestion of a diet low in protein have been studied.

No consistent, sustained increase in hemoglobin values occurred following supplementation with any of the ten "essential" amino acids or with glycine, cystine, glutamic acid, proline, or tyrosine.

These results are interpreted as evidence that no single amino acid can be regarded as a "key" amino acid in hemoglobin synthesis in the organism but rather that a combination of amino acids in as yet undetermined proportions is essential for the in vivo fabrication of the hemoglobin molecule.

LITERATURE CITED

ALBANESE, A. A., L. E. HOLT, C. N. KAJDI AND J. E. FRANKSTON 1943 Observations on tryptophane deficiency in rats — chemical and morphological changes in the blood. J. Biol. Chem., vol. 148, p. 299.

BASS, M. H. 1944 Deficiency anemia in infants. Am. J. Dis. Child., vol. 67, p. 341.

BETHELL, F. H. 1936 The blood changes in normal pregnancy and their relation to the iron and protein supplied by the diet. J. Am. Med. Assn., vol. 107, p. 564.

BLOCK, R. J., AND D. BOLLING 1943 The amino acid yield from various animal and plant proteins after hydrolysis of the fat-free tissue. Arch. Biochem., vol. 3, p. 217.

FONTES, G., AND L. THIVOLLE 1930 Action hematopoïétique du tryptophane. Action hematopoïétique de l'histidine. Comp. rend. Soc. Biol., vol. 105, pp. 965, 969.

HAHN, P. F., AND G. H. WHIPPLE 1939 Hemoglobin production in anemia limited by low protein intake. J. Exp. Med., vol. 69, p. 315.

HARRIS, H. A., A. NEUBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Biochem. J., vol. 37, p. 508.

HOGAN, A. G., E. L. POWELL AND R. E. GUERRANT 1941 Anemia from lysine deficiency in deaminized casein. J. Biol. Chem., vol 137, p. 41.

ORTEN, A. U., AND J. M. ORTEN 1943a The role of dietary protein in hemoglobin formation. J. Nutrition, vol. 26, p. 21.

——— 1943b A comparison of the hematopoietic value of certain dietary proteins. Fed. Proc., vol. 2, p. 67.

ORTEN, J. M., AND A. U. ORTEN 1944 The hematopoietic value of certain dietary proteins in the hemorrhagic anemia of the rat. Fed. Proc., vol. 3, p. 95.

ORTEN, J. M., A. U. ORTEN AND J. E. BOURQUE 1945 The inability of human or beef globin to support normal hemopoiesis in the rat without added isoleucine. Fed. Proc., vol. 4, in press.

SCHMIDT, C. L. A. 1938 Chemistry of the amino acids and proteins. Charles C. Thomas Co., Springfield, Ill, p. 217.

STURGIS, C. C., AND G. E. FARRAR 1935 Hemoglobin regeneration in the chronic hemorrhagic anemia in dogs. I. The effect of iron and protein feeding. J. Exp. Med., vol. 62, p. 457.

WHIPPLE, G. H., AND F. S. ROBSCHEIT-ROBBINS 1940 Amino acids and hemoglobin production in anemia. J. Exp. Med., vol. 71, p. 569.

EXPERIMENTAL RICKETS IN THE HAMSTER [1]

JAMES H. JONES
Department of Physiological Chemistry, University of Pennsylvania, School of Medicine, Philadelphia

(Received for publication May 8, 1945)

It is well established that the rat requires very little or no vitamin D if adequate, but not extremely excessive, amounts of available calcium and phosphorus are present in the diet. Larger animals such as man, dog, chicken, and others require this vitamin even if the diet contains sufficient quantities of calcium and phosphorus. The study of the physiology of vitamin D and its effect on calcification would be aided if it were possible to have a small laboratory animal whose needs for vitamin D would be more like that of the larger animals. With this in mind a study was made of experimental rickets in the hamster, but as the following data show this animal is similar to the rat.

EXPERIMENTAL

Two experiments were conducted with a total of 37 young hamsters which were raised in the Nutrition Fund Laboratory of Children's Hospital of Philadelphia.[2] Shortly after parturition the stock females with their young were transferred to a ration low in vitamin D. In the first experiment (21 animals) the starting age varied from 25 to 29 days and in the second experiment from 20 to 26 days. During the first week of the first experiment minor alterations were made in the vitamin contents of the diets and thereafter they remained unchanged.[3] In each of

[1] The data included in this paper were presented in abstract form in Federation Proceedings, 1945, vol. 4, p. 156.

[2] The author is grateful to Miss Claire Foster for the animals, to The Wilson Laboratories, Chicago, Ill., for the liver extract, to Mead Johnson and Co., Evansville, Ind., for the irradiated ergosterol, to Merck and Co., Rahway, N. J., for the synthetic vitamins, to Dr. C. M. McCay, Cornell University, Ithaca, N. Y., for the cellulose, and to Dr. H. M. Vars of the Harrison Department of Surgical Research, University of Pennsylvania, for the use of the photometer.

[3] The composition of the diets in grams was as follows: all diets liver extract E 2, cellulose 4, cottonseed oil 2 and wheat germ oil 1; diets 1-A, 2-A, and 3-A alcoholic extracted fibrin 18 and diets 1-B, 2-B, and 3-B alcoholic extracted fibrin 25; diets 1-A and 1-B salt mixture no. 10 (Jones, '39) 3 and diets 2-A, 2-B, 3-A, and 3-B salt mixture no. 12 (Jones and Foster, '42) 4; glucose (Cerelose) to 100 in each case; diets 3-A and 3-B 200 I.U. of vitamin D as irradiated ergosterol. The following amounts of vitamins and vitamin-containing materials were added to each 100 gm. of the above diets; SMA Carotene in oil, 5 drops; thiamine chloride, 1 mg.; riboflavin, 1 mg.; pyridoxine, 1 mg.; calcium pantothenate, 6 mg.; inositol, 6 mg.; p-amino benzoic acid, 15 mg.; choline chloride, 150 mg.; 2-methyl 1, 4-naphthaquinone, 0.05 mg.

the experiments the animals were divided into three groups. The first group was given a diet (diet 1-A) moderate in calcium, low in phosphorus (approximately 0.4% Ca and 0.02% P), and with no added vitamin D. The second group received a diet (diet 2-A) containing satisfactory amounts of calcium and phosphorus (approximately 0.6% Ca and 0.35% P) and no vitamin D. The third group received the same diet as the second group but in addition was given 200 units of vitamin D as irradiated ergosterol per 100 gm. of diet (diet 3-A). The animals of the third group (positive controls) of the first experiment did not grow so rapidly as was reported by either Cooperman, Waisman, and Elvehjem ('43) or Hamilton and Hogan ('44). Believing that this slower growth may have been due to an insufficiency of some of the essential amino acids, the protein content of the diets used in the second experiment (diets 2-A, 2-B and 2-C) was increased from 18% to 25%, but this did not improve the rate of growth. It is possible that some of the animals were below normal in regard to size at the start of the experiment as there was considerable variation in their weights at this time.

Enlargement of the wrists was detectable in the animals on diets 1-A and 1-B after 10 to 12 days, but at no time were the swellings as prominent as are frequently observed in the rat on a similar diet. With the exception of one animal on diet 1-B, which died on the twentieth day, all animals remained active and showed no definite disturbance in locomotion. Two of the animals, one on diet 1-B and one on diet 3-B developed a marked graying of the fur during the experimental period. All other animals on diets 2-A, 2-B, 3-A, and 3-B remained normal in appearance, and none on any of these latter four diets showed any enlargement of the wrists.

After 5 weeks on the experimental diets the animals were bled from the carotid artery under ether. Inorganic phosphorus was determined on 0.2 ml. of serum from each animal by a modification of the Gomori ('42) method using a Klett-Summerson photometer. Calcium was determined by the method of Clark and Collip ('25) on the pooled sera from 3 to 5 animals of the same group. The right femurs were removed and the percentage ash determined after extraction with hot alcohol. The distal ends of the ulnae and radii were removed and at least one bone from each animal was examined macroscopically, after longitudinal sectioning and staining with silver nitrate, for the extent to which rickets had developed.

The results are summarized in table 1. The first experiment is designated with the letter A and the second with the letter B. It will be noticed that there was a marked difference between groups 1-A and

1-B on the one hand as compared to groups 2-A, 2-B, 3-A, and 3-B on the other. This difference was evident in growth, level of serum inorganic phosphorus, and weight and percentage of femur ash.

In spite of the low phosphorus in the diets of groups 1-A and 1-B these animals continued to gain weight throughout the experimental period of 5 weeks, but the rate of gain was definitely less than that of the animals on the diets containing more phosphorus. The inorganic phosphorus of the sera of the animals on the phosphorus-deficient diets was very low, and additional dietary phosphorus without vitamin D (groups 2-A and 2-B) was just as effective in maintaining a normal level of this element in the serum as the giving of both phosphorus and vitamin D (groups 3-A and 3-B). There was very little difference among any of the groups in the level of serum calcium.

TABLE 1

Effect of adding phosphorus and vitamin D to low-phosphorus diets on the development of rickets in the hamster.

DIET AND GROUP NO.	NO. OF ANIMALS	GAIN IN WEIGHT	SERUM		FEMUR ASH [1]	
			Ca	P [1]		
		gm.	mg./100 ml.	mg./100 ml.	mg.	%
1-A	7	19.1	13.2	2.2 ± 0.30	13.7 ± 1.45	29.3 ± 1.78
1-B	6	24.0	11.9	2.1 ± 0.26	14.4 ± 2.98	32.0 ± 3.02
2-A	7	33.3	12.1	6.0 ± 0.27	55.2 ± 2.71	58.4 ± 0.41
2-B	5	31.4	10.7	6.7 ± 0.70	48.2 ± 2.92	55.9 ± 0.43
3-A	7	33.4	12.1	5.7 ± 0.25	55.2 ± 2.25	56.8 ± 0.58
3-B	5	39.2	12.2	5.8 ± 0.53	46.9 ± 1.11	55.4 ± 0.48

[1] Standard error of the mean follows the average.

The low serum phosphorus of groups 1-A and 1-B was reflected in both the amount and percentage of femur ash which were much below those of the animals on any of the diets containing ample phosphorus. There were no significant differences in either the absolute or relative amounts of ash in the femurs from the animals on groups 2-A and 2-B as compared to groups 3-A and 3-B.

All animals of the first two groups (1-A and 1-B) had a wide uncalcified area at the distal epiphyseal junctions of the radii. The margins of this area were, for the most part, straight and very regular, and frequently the width of the area (longitudinal direction of the bone) was greater than its length (transverse direction of the bone). The averages for these measurements were for group 1-A, width — 2.6 mm., length — 2.4 mm., and group 1-B, width — 2.1 mm., length — 1.9 mm.

The corresponding uncalcified areas in all of the animals of the other four groups were very narrow and macroscopically appeared normal.

In every respect the animals on the diets containing sufficient quantities of calcium and phosphorus and no vitamin D were as free from the usual manifestations of rickets as were the animals on the same diets but given vitamin D. In contrast to this the animals on the diets low in phosphorus and moderate in calcium and free from vitamin D showed all the customary signs of experimental rickets as seen in the rat on a similar diet. In these experiments, however, the hamster grew better than is usual for the rat on diets so low in phosphorus, growth was maintained longer, less difficulty with locomotion was observed and there was less enlargement at the wrists. Serum phosphorus and bone ash, on the other hand, were as low as that generally seen in the rat, and compared to the diameter of the bones the width of the uncalcified areas at the epiphyses of the radii was greater. This latter was probably the result of the superior growth and the longer duration (5 weeks) of the experiments.

SUMMARY AND CONCLUSIONS

On a diet containing approximately 0.4% calcium and 0.02% phosphorus with no added vitamin D hamsters developed all of the symptoms characteristic of experimental rickets observed in the rat on similar diets. Calcification was as rapid on a diet containing optimal amounts of calcium and phosphorus and no vitamin D as on the same diet to which the vitamin had been added.

Typical rickets can be produced in the hamster, but as with the rat the diet must be low in phosphorus as well as deficient in vitamin D.

LITERATURE CITED

Clark, E. P., and J. B. Collip 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

Cooperman, J. M., H. A. Waisman and C. A. Elvehjem 1943 Nutrition of the golden hamster. Proc. Soc. Exp. Biol. and Med., vol. 52, p. 250.

Gomori, G. 1942 A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med., vol. 27, p. 955.

Hamilton, J. W., and A. G. Hogan 1944 Nutritional requirements of the Syrian hamster. J. Nutrition, vol. 27, p. 213.

Jones, J. H. 1939 A comparison of cereal and non-cereal diets in the production of rickets. J. Nutrition, vol. 18, p. 507.

Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, vol. 24, p. 245.

THE EFFECT OF FAT UPON THE UTILIZATION OF GALACTOSE BY THE RAT [1]

L. P. ZIALCITA, JR.,[2] AND H. H. MITCHELL
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication May 10, 1945)

Schantz, Elvehjem and Hart ('38) have observed an interesting relationship between fat and the utilization of lactose in milk. They found that when weanling albino rats were placed on a mineralized skimmed milk diet, reducing sugar was readily detected in the urine after a few days of feeding. The sugar was identified as galactose and accounted for all the reducing material in the urine — as high as 35% of the ingested galactose. When butterfat, lard, corn oil, linseed oil, or palmitic and oleic acids were added to the mineralized skimmed milk at levels of 3 to 4%, this loss was prevented. Similar experiments were carried out using dry synthetic rations, and here also the addition of fat to the lactose-containing ration to the extent of 35% stopped the loss of most of the sugar but not all.

In a later paper (Schantz and Krewson, '39), it was reported that synthetic even-chain fatty acids containing twelve or more carbon atoms were equally effective in the prevention of the galactose excretion in the urine. This was not true with acids containing less than twelve carbon atoms, nor those with odd-chains. The addition of glucose to the diet at levels of 8 and 10% did not prevent the loss, although a small decrease was observed. Mitchell, Merriam and Cook ('37) reported a galactosuria of varying degree in rats on high-lactose or high-galactose diets containing 11% of fat.

EXPERIMENTAL

In this study 30 weanling albino rats were equally distributed into 10 trios. The basal ration consisted of 48% lactose, 20% ether-extracted casein, 28% fat or glucose, and 4% salt mixture. This diet was amply supplemented with vitamins in pure form. The first rat in each trio

[1] The data reported in this paper were taken from a thesis presented by Mr. L. P. Zialcita, Jr., to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy.

[2] Fellow, University of the Philippines, Manila.

received butterfat; the second, corn oil; and the third, glucose. All rats in each trio with few exceptions consumed equal amounts of their respective diets and isocaloric intakes were maintained by the addition to the diet of the third rat in each trio of proper amounts of a glucose solution.

The animals were placed in separate metabolism cages, designed so that the urine and the feces were collected separately and quantitatively. The urine was collected under toluene. The diets were so offered that spilling was prevented. The collection period extended for 3 days and began when the rats had been on their respective diets for not less than 2 weeks.

Galactose in the urine was determined by a slight modification of the methods of Hoffman ('37) and of Morell ('41). Glucose was eliminated from the urine samples by fermentation with Saccharomyces cerevisiae, while other possible reducing substances were removed by treatment with Lloyd's reagent. The galactose samples were read against a standard curve (prepared with anhydrous d-galactose, Pfanstiehl) in a Coleman Universal Spectrophotometer at 425 mµ.

The accuracy of the method used, as well as of the collection of the urine and its sampling, was attested by recovery trials of pure and added galactose involving the whole procedure. These values ranged from 94 to 102%. The specificity of the method for galactose was verified with the help of two yeasts,[3] Saccharomyces bayanus NRRL Y-996 (which ferments glucose but has no action on galactose) and Saccharomyces carlsbergensis NRRL Y-379 (which ferments both glucose and galactose).

EXPERIMENTAL RESULTS

The average galactose excretions on the three rations studied are given in table 1.

With an intake that varied from 3.36 to 5.04 gm. of galactose daily per rat, the average daily urinary excretion expressed in per cent of the amount ingested was as follows: For the butterfat group, 30.7 with a standard error of $\pm$ 2.18; for the glucose group, 28.6 $\pm$ 2.16; and for the corn oil group, 20.7 $\pm$ 1.07. Statistical treatment of the mean differences between the three groups (using Fisher's "t" test) revealed no significant difference between the butterfat and the glucose group (P[4] = 0.5), but clearly significant differences between the butterfat

[3] Kindly furnished by the U. S. D. A. Northern Regional Research Laboratory, Peoria, Illinois.

[4] This and subsequent values of P give the probability on a scale of 1.0 that a random combination of the uncontrolled factors in the experiment would of itself cause a difference in the mean values compared as great or greater than that observed. If P is less than 0.05 in the Fisher, or 0.025 in a Student test, it may reasonably be neglected.

and the corn oil rats ($P = <0.01$) and between the glucose and the corn oil rats ($P = <0.01$). Application of "Student's" method ('25) to paired rats for which the galactose intake was the same confirmed the previous analysis in showing no difference between the rats receiving butterfat and those receiving glucose ($P = 0.38$), but significant differences between the rats receiving butterfat and those receiving corn oil ($P = 0.008$), and between those receiving glucose and those receiving corn oil ($P = 0.004$).

TABLE 1

The intake and urinary loss of galactose, with and without dietary fat.

TRIO NO.	RATS ON BUTTERFAT			RATS ON CORN OIL			RATS ON GLUCOSE		
	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose
	mg.	mg.	%	mg.	mg.	%	mg.	mg.	%
1	3600	946.6	26.3	3600	833.3	23.1	3600	788.3	21.9
2	3600	1155.0	32.1	3600	728.0	20.2	3600	950.0	26.4
3	3360	640.5	19.1	4320	1033.3	23.9	4320	1236.6	28.6
4	4320	1621.6	37.5	4320	540.8	12.5	3120	596.6	19.1
5	3600	1233.3	34.3	3120	732.4	23.5	3600	1476.6	41.0
6	3600	781.6	21.7	3600	648.2	18.0	3600	1253.4	34.8
7	4320	1486.6	34.4	4320	950.0	22.0	4320	1326.7	30.7
8	5040	1358.3	27.0	5040	1130.0	22.4	5040	1536.7	30.5
9	5040	2015.0	40.0	5040	1010.0	20.0	5040	1621.7	32.2
10	5040	1750.0	34.7	4560	986.6	21.6	5040	1030.0	20.4
Averages			30.7			20.7			28.6

Corn oil, but not butterfat, under the conditions of this experiment, decreased the loss of galactose in the urine by some 27%.

In the first two of the previously cited studies, the diet involved in most of the tests was fresh mineralized skimmed milk to which various fats had been added. In the present study an artificial solid milk diet was employed. The amounts of fat or glucose, and the lactose involved were about the same when converted to a dry basis. There is therefore no obvious explanation for the divergence of the results obtained. It would appear that the galactose excretion observed in this study was merely the result of an alimentary galactosuria present in all three groups, though to a somewhat smaller extent when corn oil, but not butterfat, was incorporated in the diet. Obviously this is not a fat effect. Attention may be called to the fact that Mitchell, Cook and Mer-

riam ('37) found that fats as such did not appreciably alter the development of cataract in rats consuming diets high in lactose or galactose, even when the fat constituted 46% of the diet. Fats, as such, do not seem to be concerned in the metabolism of galactose.

CONCLUSION

The results obtained in this study fail to confirm the reported influence of fat, as such, on the metabolism of galactose. Neither is there any reasonable expectation from the available facts of animal nutrition that dietary fats should favor the utilization of galactose in animal metabolism.

Under the conditions of this experiment, corn oil, but not butterfat, decreases the urinary loss of galactose on a diet containing 48% lactose, by about one-fourth. This may be an effect of some non-glyceride constituent of corn oil.

LITERATURE CITED

HOFFMAN, W. S. 1937 A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem., vol. 120, p. 51.

MITCHELL, H. S., G. M. COOK AND O. A. MERRIAM 1937 The specificity of galactose as a cataract producing agent. J. Nutrition, vol. 13 (suppl.), p. 18.

MITCHELL, H. S., O. A. MERRIAM AND G. M. COOK 1937 The relation of ingested carbohy drate to the type and amount of blood and urine sugar and to the incidence of cataract in rats. J. Nutrition, vol. 13, p. 501.

MORELL, S. A. 1941 Rapid determination of reducing sugars. Extention of Forsee's photocolorimetric ferricyanide method. Ind. Eng. Chem., Anal. Ed., vol. 13, p. 249.

SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1938 The relation of fat to the utilization of lactose in milk. J. Biol. Chem., vol. 122, p. 381.

SCHANTZ, E. J., AND C. F. KREWSON 1939 Further studies on the relation of fat to utilization of lactose in milk. Proc. Soc. Exp. Biol. Med., vol. 42, p. 577.

"STUDENT" 1925 New tables for testing the significance of observations. Metron, vol. 5, p. 105.

THE REQUIREMENT OF THE TURKEY POULT FOR VITAMIN B_c[1]

LUTHER R. RICHARDSON, ALBERT G. HOGAN AND HARRY L. KEMPSTER
Departments of Agricultural Chemistry and Poultry Husbandry,
University of Missouri, Columbia

ONE FIGURE

(Received for publication June 7, 1945)

Several feedstuffs, such as dried skim milk or alfalfa leaf meal, are regarded as important or even critical items in the ration of newly hatched turkey poults, but in recent years the supply has not been equal to the demand. An attempt was made in this laboratory to formulate satisfactory rations that contained none of these constituents, and although some degree of success was attained the poults made only moderate gains in weight. It was suggested that the slow rate of growth was due to an amino acid deficiency, and, to test this hypothesis, the vegetable protein concentrate in one of the practical rations was replaced by casein and gelatin. When this change was made the ration was a complete failure and the poults assumed a typical, and abnormal, posture. Such results led us to a more extended study of the problem.

EXPERIMENTAL

Day-old Bronze turkey poults were transferred to small brooders provided with automatic electric heating units. The temperature varied from 90° to 95°F. At the age of 2 weeks they were transferred to another room, maintained at a temperature of 80° to 85°F. The experimental rations, and water, were constantly available. It may be of some interest to mention here that practical turkey growers often state that poults must be taught to eat, but in our experience this is unnecessary. If the environment and ration are suitable, and if the feeders are properly constructed, normal poults begin to eat promptly. The poults were weighed once a week, and they were examined frequently for abnormalities.

[1] Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series no. 980. Aided by a grant from Parke, Davis and Company.

The ration in use when this type of paralysis first appeared was modified slightly in later trials, but most of the modifications had no significance and for the present only Ration A will be described. While these studies were under way we were also investigating the response of turkey poults to synthetic diets. Poults grow remarkably well on these diets when a liver extract is included, and therefore various liver fractions were added to the synthetic diets in an attempt to attain

TABLE 1

Rations deficient in vitamin B_c.

CONSTITUENTS	RATION A	RATION B
	gm.	*gm.*
Yellow corn	49.50	
Wheat bran	5.00	
Wheat middlings	15.00	
Steamed bone meal	2.00	
$CaCO_3$	1.00	
NaCl	0.46	
$MnSO_4, 4H_2O$	0.04	
Casein	20.00	35.00
Gelatin	5.00	10.00
Cerelose		32.00
Cellulose		3.00
Lard [1]	2.00	10.00
Mineral salts		5.00
Liver extract fraction B [2]		5.00
Additional vitamins	see footnote [3]	see footnotes [3] [4]

[1] Carrier for fat-soluble vitamins.

[2] This fraction contains only a trace of vitamin B_c.

[3]

Vitamin A	2000 I.U.	Alpha-Tocopherol	2.5 mg.
Vitamin D	283 A.O.A.C Units	2-methyl-1, 4-napthoquinone	2.5 mg.
Thiamine	1 mg.	Ca-Pantothenate	2.0 mg.
Riboflavin	1 mg.	Nicotinic acid	5.0 mg.
Pyridoxine	1 mg.	Choline	200.0 mg.

[4] Inositol 100 mg., p-aminobenzoic acid 100 mg., biotin 10 μg.

All vitamins except A and D were supplied by Merck and Co., Rahway, N. J.

greater concentration of the active agent. One [2] of the extracts was described to us as the filtrate, or by-product, from the first stage in the isolation of vitamin B_c (Pfiffner et al., '43). This fraction contains only a trace of vitamin B_c as indicated by microbiological assay, and when it was substituted for the original liver extract, as in Ration B, the poults developed cervical paralysis even more quickly than they did on Ration A. The composition of the two rations is shown in table 1 and

[2] Provided by Dr. J. J. Pfiffner, of Parke, Davis and Company.

the observations on the poults are summarized in table 2. Several abnormalities developed, but the most characteristic symptom was a spastic cervical paralysis. At the onset the neck is extended and rigid, as in a turkey that has been alarmed and is searching for the source of danger. The attacks are at first intermittent, but later they become continuous. The wings are slightly drooped and quiver, the poult chirps continuously as if in pain, and there may be some diarrhea with the excreta thin and white. The condition terminates in death within 24 to 36 hours unless vitamin B_c is provided during the early stages.

There were ten poults in the first trial with Ration A, and it will be noted that five of them developed cervical paralysis. None of these recovered and all of them died in a day or two. Four more died with no

TABLE 2

Vitamin B_c deficiency in turkey poults.

RATION	POULTS PARALYZED			POULTS NOT PARALYZED					
				Died			Survived		
	No.	Age	Wt.	No	Age	Wt.	No.	Age	Wt.
		weeks	*gm.*		*weeks*	*gm.*		*weeks*	*gm.*
A 1	5	7	765	4	4	81	1	14	1880
2	15	3.5	240	12	2.5	153	4	6	552
B	9	2.5	143	2	2	102	0		
B plus B_c conc.	0			0			2	4	498

[1] The mortality is 100% unless vitamin B_c is provided.

characteristic symptoms, and one survived for 14 weeks with no symptoms.

In subsequent trials there were thirty-one poults, and fifteen of them developed the characteristic symptoms. None of these survived except a few which were treated in the early stages with some source of vitamin B_c. Twelve died without characteristic symptoms, and four survived for 6 weeks when they were discarded. It will be noted that in trial 1 the poults developed the symptoms at a later age than in trial 2, presumably because the mothers of the poults had consumed different types of rations. The poults in trial 1 were hatched in October, and their mothers had probably been on range of some kind and had consumed a considerable quantity of fresh forage, which we believe is at least a fair source of vitamin B_c. The poults in trial 2 were hatched in the early spring, and one might assume that the hens that laid the eggs from which they were

hatched had not had access to fresh forage for some weeks. If this explanation is correct the poults were partly depleted of vitamin B_c when hatched. The rate of growth on Ration A was lower than that of poults on our better rations but it was not markedly subnormal.

Two poults were given Ration B with a supplement of a highly concentrated preparation of vitamin B_c.[2] They grew at a normal rate but more extensive studies must be carried out before one can be certain

TABLE 3

Curative treatment of cervical paralysis.

RATION	TREATMENT		NUMBER TREATED	NUMBER THAT RECOVERED	NUMBER DYING AFTER RECOVERY
A (and modifications)	Yeast and milk		2	2	2
	Dried yeast	2 gm.	1	0	
	Yeast eluate	1 gm.	3	2	
	B_c concentrate	105 µg.	1	0	
		210 µg.	4	2	
	Crystalline vitamin B_c	100 µg.	4	1	
		300 µg.	1	0	
	Intraperitoneal injection of crystalline vitamin B_c	50 µg.	2	2	
		100 µg.	2	2	
B	B_c concentrate	210 µg.	3	2	1
	Crystalline vitamin B_c	100 µg.	1	1[1]	
	Crystalline vitamin B_c, 50, 100, and 50 µg. on successive days		1	1[2]	
	Total		25	15	3

[1] Recurrence in 6 days.
[2] Recurrence in 7 days.

that their diet was entirely adequate. Our experience with the concentrate gave no reason to infer that it contained any vitamin other than B_c which is required by the poult. This inference is supported by the fact that chicks which received Ration B supplemented with crystalline vitamin B_c[2] grew at a rate which exceeds that of any current growth standard for the breed employed. Eleven poults received Ration B alone, with no supplements, and nine of them developed cervical paralysis, at an average age of 2.5 weeks. The other two died with no specific symptoms. The results of the various curative treatments are described in table 3.

[2] Provided by Dr. J. J. Pfiffner, of Parke, Davis and Company.

Table 3 presents the results of the curative treatments. Two poults were given a suspension of yeast in milk and both recovered, but they died not long afterward. It is assumed that the suspension contained enough vitamin B_c to give temporary relief, but not enough to restore the poults to a normal physiological condition. One poult was given 2 gm. of dried yeast but it did not recover. Three poults received an eluate of a fuller's earth adsorbate of yeast extract, and two recovered. Eight poults received the vitamin B_c concentrate, and four recovered. Eleven poults were treated with the crystalline vitamin and seven recovered. It will be noted that one of these received three doses, on 3 successive days, before it recovered completely. It is possible that some of the other four would have survived if they had been treated with similar repeated doses. Four of the eleven received the vitamin by intraperitoneal injection and all four recovered. Of the two poults which received 100 µg. of vitamin B_c by intraperitoneal injection, one recovered in 4 hours, the other in 6 hours. A third poult received 50 µg. at 10:00 A.M. There was marked improvement at 5:00 P.M. but the symptoms of deficiency had not entirely disappeared. Recovery was complete by the next morning. The fourth poult received the same quantity of the vitamin at 2:45 P.M. There was no definite improvement at 5:00 P.M. but it was normal the next morning. We are of the opinion that the vitamin is more effective when injected than when it is administered orally, and that curative treatment under our experimental conditions is entirely effective if initiated promptly but less effective when delayed. There were no cases of spontaneous remission, and the cures induced by the crystalline preparation leave no doubt that the disease was caused by a deficiency of vitamin B_c.

According to Hogan and Parrott ('40) and O'Dell and Hogan ('43) chicks become anemic when they consume diets that are deficient in vitamin B_c; therefore red cell volume was determined in some of the affected poults. The volume of erythrocytes tended to be low, but none of the poults was markedly anemic.

A typical example of a B_c deficiency is illustrated in figure 1. This same type of paralysis has been observed occasionally in chicks which survive for some time after they become anemic.

Distribution of vitamin B_c

Our data on this point are limited, but it is clear that the vitamin is present in some of the feedstuffs in only small quantities. Ration A for example contains 51.5% of yellow corn, 5% of wheat bran, and 15% of

wheat middlings, but some poults, when fed this mixture, gave evidence of a deficiency within 3 weeks. A considerable number of poults received a ration, not previously mentioned, which contained 22% of yellow corn, 3% of wheat bran, 9% of wheat middlings, and 15% of soybean meal, and approximately 8% of these poults developed deficiency symptoms, at ages varying from 15 to 24 days. Soybean meal is not an excellent source of vitamin B_c but it probably contains more than is present in corn or the wheat by-products. It has been observed (unpublished) that anemia in chicks is delayed, but not prevented, by including 2% of high grade alfalfa meal in the ration.

Diet B — Age, 4 weeks — Weight, 202 gm.
Adequate diet — Age, 4 weeks — Weight, 510 gm.

Fig. 1 The posture of the poult which received Diet B is typical of a deficiency of vitamin B_c.

One poult, not described in this report, developed typical symptoms and died on a simplified ration which contained 5% of dried Strain G yeast. One may reasonably assume that vitamin B_c deserves some consideration in practical turkey production.

SUMMARY

Turkey poults develop a spastic type of cervical paralysis on rations that are severely deficient in vitamin B_c, but which contain reasonable amounts of all other required vitamins.

The poults which were examined had a lower red cell volume than is normal but none were markedly anemic.

Remission of the symptoms was obtained by the administration of crystalline vitamin B_c.

Some of the commonly used feedstuffs are poor sources of vitamin B_c.

LITERATURE CITED

HOGAN, A. G., AND E. M. PARROTT 1940 Anemia in chicks caused by a vitamin deficiency. J. Biol. Chem., vol. 132, p. 507.

O'DELL, B. L., AND A. G. HOGAN 1943 Additional observations on the chick antianemia vitamin. J. Biol. Chem., vol. 149, p. 323.

PFIFFNER, J. J., S. B. BINKLEY, E. S. BLOOM, R. A. BROWN, O. D. BIRD, A. D. EMMETT, A. G. HOGAN AND B. L. O'DELL 1943 Isolation of the antianemia factor (vitamin B_c) in crystalline form from liver. Science, vol. 97, p. 404.

VITAMIN A AND CAROTENE IN THE NUTRITION OF THE GUINEA PIG[1]

LILLIAN S. BENTLEY AND AGNES FAY MORGAN
Laboratory of Home Economics, University of California, Berkeley

ONE FIGURE

(Received for publication April 23, 1945)

The nutritional needs of the guinea pig are still largely unknown in spite of much effort in several laboratories to determine them. The purified diets used for rats and dogs are not satisfactory for guinea pigs, and even the semipurified diets frequently used are only partly successful.[2] To study the guinea pig's response to vitamin A and carotene, the diet must be free of both compounds; thus a suitable semipurified diet had to be devised. In this investigation, therefore, it was necessary first to obtain a diet that was adequate when supplemented with vitamin A, and second to establish a means of recognizing the deficiency in the guinea pig.

Boock and Trevan ('22) investigating the cause of the death of guinea pigs on a diet of bran, oats, mangolds, and water found great improvement in all except one animal when they were given 0.5 ml. of cod liver oil. On a diet of bran and oats, caseinogen, salts, paper and mangolds, two guinea pigs began to lose weight at the end of a month and one developed a cloudiness of the cornea and keratomalacia. Wolbach and Howe ('28) used a diet of casein 15%, starch 74%, butterfat 6%, salts 3%, and yeast 2%; filter paper and distilled water ad libitum, supplemented by orange juice. When lard was substituted for the butter and the amount of orange juice reduced, the only external signs of vitamin A deficiency were cessation of growth and loss of weight. The animals died before typical xerophthalmia was produced.

Though Manzi ('35) attributed loss of hair, eczema of the face, and abortion to vitamin A deficiency, xerophthalmia was observed in only one of his twenty-three guinea pigs. The diet used may have been deficient in other factors, possibly some of the B vitamin complex, and

[1] This study was supported by a grant from Swift and Co., Chicago.

[2] It has been reported that guinea pigs require three unknown dietary essentials (Woolley '42). No attempt was made in this study to verify these claims or to identify the unknowns.

VITAMIN A AND CAROTENE IN THE NUTRITION OF THE GUINEA PIG[1]

LILLIAN S. BENTLEY AND AGNES FAY MORGAN
Laboratory of Home Economics, University of California, Berkeley

ONE FIGURE

(Received for publication April 23, 1945)

The nutritional needs of the guinea pig are still largely unknown in spite of much effort in several laboratories to determine them. The purified diets used for rats and dogs are not satisfactory for guinea pigs, and even the semipurified diets frequently used are only partly successful.[2] To study the guinea pig's response to vitamin A and carotene, the diet must be free of both compounds; thus a suitable semipurified diet had to be devised. In this investigation, therefore, it was necessary first to obtain a diet that was adequate when supplemented with vitamin A, and second to establish a means of recognizing the deficiency in the guinea pig.

Boock and Trevan ('22) investigating the cause of the death of guinea pigs on a diet of bran, oats, mangolds, and water found great improvement in all except one animal when they were given 0.5 ml. of cod liver oil. On a diet of bran and oats, caseinogen, salts, paper and mangolds, two guinea pigs began to lose weight at the end of a month and one developed a cloudiness of the cornea and keratomalacia. Wolbach and Howe ('28) used a diet of casein 15%, starch 74%, butterfat 6%, salts 3%, and yeast 2%; filter paper and distilled water ad libitum, supplemented by orange juice. When lard was substituted for the butter and the amount of orange juice reduced, the only external signs of vitamin A deficiency were cessation of growth and loss of weight. The animals died before typical xerophthalmia was produced.

Though Manzi ('35) attributed loss of hair, eczema of the face, and abortion to vitamin A deficiency, xerophthalmia was observed in only one of his twenty-three guinea pigs. The diet used may have been deficient in other factors, possibly some of the B vitamin complex, and

[1] This study was supported by a grant from Swift and Co., Chicago.

[2] It has been reported that guinea pigs require three unknown dietary essentials (Woolley '42). No attempt was made in this study to verify these claims or to identify the unknowns.

the eczema and loss of hair could have been the result of such deficiency. Furthermore the animals used had passed their most rapid growth period, and thus would have been less susceptible to a deficiency of vitamin A. Hetler ('34) fed guinea pigs the vitamin-A free diet used in her study of the deficiency in monkeys. Three of the guinea pigs died within a month without developing eye lesions. Three others that had received this diet plus rolled oats ad libitum lived 6 to 9 weeks but, during the last 2 or 3 weeks lost weight rapidly and developed eye lesions. The symptoms observed were dryness and scaliness of the eyelids and cloudiness of the corneas. It was concluded that the guinea pig may react to the deficiency in the manner observed in other species.

Since in all other species studied a deficiency of vitamin A produces xerophthalmia, it seemed unlikely that typical xerophthalmia could not be produced in guinea pigs by means of a vitamin-A-deficient regime. Therefore the attempt was made to verify this supposition.

METHODS

Six semipurified diets and a stock diet were employed in studying vitamin A deficiency, the storage of vitamin A, the utilization of carotene for vitamin A storage, and the adequacy of the preformed vitamin and provitamin during reproduction.

The stock diet

The diet used for the guinea pig colony was a commercial feed [3] composed of ground grains, bran, molasses, soy bean, alfalfa, linseed and bone meals, salt and dried whey. This feed when supplemented with ascorbic acid-fortified orange juice and carotene in oil had been found satisfactory for both growth and reproduction.

The semi-purified diets

The simplified diet, diet C, eventually used, contained, in per cent, casein 25, cornstarch 40, hydrogenated cottonseed oil 3.5, salts [4] 4, K_2CO_3 0.5, wheat germ 10, yeast 10 and bran 7. When supplemented with 6 ml. lemon juice containing 1 mg. ascorbic acid per milliliter three times a week, and with an adequate amount of carotene or vitamin A this diet allowed normal growth of young guinea pigs. Diets I and II were

[3] Globe A — 1 rabbit pellets; Globe Grain and Milling Co., Oakland, California. The composition was as follows: crude protein 15, fat 2.5, fiber 19, ash 10, added salts 1, carbohydrates 52.5%, respectively; thiamine 0.28, riboflavin 0.65 and carotene 0.58 mg. %, respectively.

[4] Hubbell, Mendel, and Wakeman ('37).

tried earlier. These contained larger and smaller proportions of casein (30 and 20% and a lower level of salts (2% with no additional K_2CO_3)). These diets proved inadequate, resulting in poor consumption and failure of growth. Daily addition of 1 ml. cottonseed oil alone or containing 1 mg. mixed tocopherols [5] did not improve the performance on these diets but the increase in salt content from 2 to 4.5% allowed normal growth in the young guinea pigs, 31 gm. gain weekly for 6 weeks (fig. 1).

The modification of diet C, aimed at making it low in vitamin A by use of hot-alcohol extracted casein, was designated diet G. This change in the casein was sufficient to bring about deficiency signs and death when the animals were transferred from C to G. Two other modifications were used, in that carotene-free grass [6] residue or additional cornstarch were substituted for the wheat germ in the diets designated A and B. A concentrated carotene-free water extract of the dried grass was also fed along with these diets at first but these grass preparations were found to have no effect upon growth and were later discarded. The vitamin A supplement used was a solution of the vitamin [7] in cottonseed oil; the carotene was a carotene-oil concentrate [8] which was assayed for vitamin A value by the U.S.P. biological procedure.

Procedure

The animals were, for the most part, produced in the laboratory colony. They were caged in groups or reared in open runs on shavings. Most of the guinea pigs were placed on the experimental diets when they weighed 200 to 300 gm., at 3 to 5 weeks of age.

The vitamin A content of the livers was determined by the method of Davies ('33) with a few minor changes. The blue color developed with the reagent (Koehn and Sherman, '40) was read in an Evelyn photoelectric colorimeter, and the vitamin values were calculated by comparison with standard curves for crystalline vitamin A.[9] The serum vitamin A was determined by the method of Yudkin ('41). The volume

[5] Concentrate of natural mixed tocopherols, 40%. This was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

[6] Cerophyl, produced by Cerophyl Laboratories, Inc., Kansas City, Missouri.

[7] This was distilled from fish liver oil and was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y. The oil contained 150 mg. of vitamin A per gram as determined chemically.

[8] This concentrate, a product of General Biochemicals, Inc., Chagrin Falls, Ohio, contained 2 mg. carotene per ml.

[9] This was generously supplied as vitamin A alcohol by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

was adjusted for reading with the 6 ml. window of the Evelyn photoelectric colorimeter.

SYMPTOMS OF VITAMIN A DEFICIENCY

The signs of deficiency developed within 2 to 3 weeks. Weight loss nd sensitivity of the eyes to light were the first symptoms. Swelling nd incrustation of eyelids, keratinization, and corneal destruction by nfection were seen in the animals that survived 4 to 6 weeks.

Unless the symptoms of the deficiency were recognized early and herapy instituted at once, the animals could not be saved. Four of five animals died shortly after an attempt was made to cure the deficiency by giving vitamin A or carotene. One of those in the first group (209, ig. 1) responded to the therapy. After the symptoms had been cleared

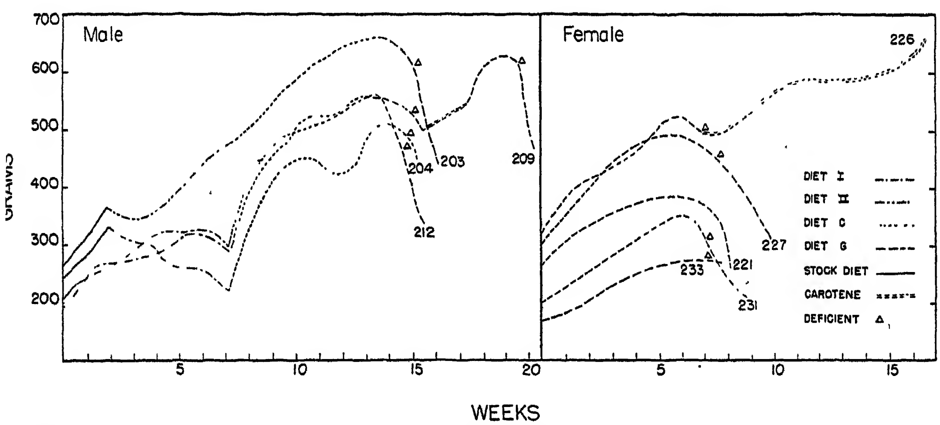


Fig. 1 The growth of male and female guinea pigs on semipurified diets with and without carotene.

up the carotene supplement was withdrawn in order to determine how long the animal could maintain itself on the amount of carotene that had been given (9.74 mg.). This animal succumbed 3 weeks after cessation of the carotene medication. Although there were a few necrotic areas on the liver that were indicative of infection, the evidence pointed to an old infection rather than an active one. Since there was incrustation of the eyes and the cornea of one eye was opaque, the animal was considered to have died in a state of vitamin A deficiency.

STORAGE OF VITAMIN A FROM DIETARY CAROTENE AND VITAMIN A

A group of male guinea pigs from the stock colony, weighing 200 to 300 gm. each, were weaned to diets A and B (table 1). One pair of ani-

mals on diet B received the supplement of lemon juice only, while the other pair received in addition to lemon juice the carotene-free grass concentrate. When the first symptoms of vitamin A deficiency appeared, one guinea pig in each pair received vitamin A, the other carotene. After the deficiency symptoms had cleared up, the vitamin A and carotene feeding was discontinued to see how long the guinea pig could maintain itself on the stores built up from the amount of vitamin or provitamin given. As soon as the symptoms reappeared the animals were again fed the vitamin or the provitamin, then sacrificed after a period and the livers analyzed. The guinea pigs that received 10 mg. carotene became depleted on the average in 21 days when the supplement was discontinued, whereas those that received 3 mg. of vitamin

TABLE 1

Repeated depletion of male guinea pigs followed by the feeding of vitamin A or carotene.

BASAL DIET	GUINEA PIG NUMBER	TOTAL SUPPLEMENT IN 20 DAYS AFTER FIRST DEPLETION	PERIOD REQUIRED FOR SECOND DEPLETION	SUPPLEMENT FED AFTER SECOND DEPLETION	VITAMIN A IN LIVER
			days		*µg.*
A	223 [1]	Carotene, 10 mg.	20	Carotene, 2 mg. in 4 days	0
B	229 [1]	Carotene, 10 mg.	23	Carotene, 11.5 mg. in 23 days	trace
B	222	Carotene, 10 mg.	20	Carotene, 11.5 mg. in 23 days	0
A	230 [1]	Vitamin A, 3 mg.	32	Died of hemorrhage, no supplement	0
B	220 [1]	Vitamin A, 3 mg.	32	Vitamin A, 1.9 mg. in 13 days	39
B	219	Vitamin A, 3 mg.	27	Vitamin A, 3.6 mg. in 24 days	213

[1] Received carotene-free dried-grass concentrate equivalent to 1 gm. of dried grass weekly.

A were depleted in 31 days. All the guinea pigs required 20 days for depletion when they were first placed on the diet. This was after the suckling period during which they had eaten the stock diet supplemented with orange juice, as well as the mother's milk, that is, a diet in which the source of vitamin A was chiefly carotene.

In general, no vitamin store was detectable in the carotene-fed animals, yet in spite of this, it took 20 days to deplete these animals and 30 days for the animals that received vitamin A. Judging by the liver values obtained after the second period of supplementary feeding, one must assume that the animals fed vitamin A had some vitamin A stored in the liver which they could draw upon during depletion.

Apparently carotene in amounts ranging from 2 mg. in 4 days to 11.5 mg. in 23 days was inadequate for production of significant stores of vitamin A, but vitamin A, 1.9 to 3.6 mg. in 13 or 24 days allowed the formation of such stores.

The possibility remained that the carotene itself might be stored to some extent in the liver. A depletion period of only 20 or 30 days, however, is what one would expect to find with the vitamins that the animal does not store but requires on a daily-intake basis, for example, the water-soluble vitamins. This period is comparable with that required for the depletion of ascorbic acid, which is not considered to be one of the vitamins effectively stored. Even the depletion period for those animals that received the vitamin and presumably stored it was relatively short.

The site of vitamin A storage

Likewise it seemed that there might also exist small undetected stores of liver vitamin A in the carotene-fed animals, or that other storage sites exist. In order to test the latter hypothesis five young animals were used. Three of these had been fed on fresh green grass ad libitum, one had received the distilled vitamin A and one had received the carotene concentrate for several weeks preceding sacrifice. The whole carcasses of the latter two animals, exclusive of the liver and the blood serum, were digested with KOH and the unsaponifiable fraction of the oil tested with the Carr-Price reagent. The liver and serum were examined separately. No vitamin A was found in the carcasses or in the liver of the carotene-fed animal but 150 μg. in the liver of the vitamin A fed guinea pig and 40 μg. per 100 ml. serum. The carotene-fed animal had 22 μg. per 100 ml. serum.

The three grass-fed guinea pig specimens were pooled and kidneys, lungs, adrenals, hearts, washed gastro-intestinal tracts, testes, as well as livers, sera and remaining carcasses were examined separately. No vitamin A was found except in sera, livers and a trace in the kidneys. The totals were 33 μg. per 100 ml. sera, 1850 μg. in the three livers, and 5 μg. in the combined kidneys.

Rate of depletion of vitamin A stores

The range and rate of depletion in apparently healthy young animals were investigated. Sixteen guinea pigs purchased from various commercial growers were maintained on a diet of alfalfa hay and rolled barley with carrots fed twice weekly for several weeks. The guinea pigs were then sacrificed and the livers analyzed for vitamin A. Since these animals had been purchased from various growers the early dietary history varied. The diet generally used by growers consists of oat hay, rolled barley, alfalfa (pellets or hay), greens or green vegetable tops,

and sometimes carrots. Only one guinea pig out of sixteen had a measurable amount of vitamin A in its liver; one other showed a trace. This seemed to indicate even poorer utilization of carotene as a source of vitamin A for storage than was found by Chevallier and Choron ('35, '36). Clausen and McCoord ('34) reported that 1 mg. of carotene daily for 2 weeks did not increase the vitamin A content of the guinea pig's liver.

Of the group of female guinea pigs used for depletion studies some weighed 250 to 300 gm., and some that were older weighed 340 to 480 gm. Five were placed on diet G. Four of the animals maintained on diet G did not receive vitamin A and manifested symptoms of vitamin A deficiency. They died shortly after the appearance of the deficiency-symptoms and no vitamin A was found in the livers. The fifth animal, 226, was given 0.5 mg. carotene in 0.25 ml. oil daily a week after deficiency signs appeared. At the end of 31 days the amount of carotene given was doubled. This guinea pig received a total of 53.5 mg. carotene in 69 days after having been depleted to the deficient stage (fig. 1), but had a store of only 50 μg. vitamin A in the liver. Four female guinea pigs, littermates of the animals placed on diet G, were placed on stock diet. After the growth curve was established for the four animals on the stock diet, which contained 0.58 mg. carotene per 100 gm., they were fed different forms of carotene for 38 days to determine the extent of the vitamin storage. Two were given 1.0 mg. carotene in dried grass (cerophyl) daily, plus 1.0 ml. of cottonseed oil; the other two received the same amount of carotene, 1.0 mg. daily, in the form of 1.0 ml. of a carotene oil concentrate. For guinea pigs weighing 500 gm., this amount was equivalent to 2.0 mg. per kilogram of body weight daily, which is sixteen times the amount reported as necessary for significant storage in the other mammals that have been studied (Guilbert, Howell and Hart, '40). Chemical analysis of the livers revealed a small amount of vitamin A in all the livers. The values ranged from a trace to 0.8 μg. per gram and the maximum total storage was only 23 μg. for the entire liver. This is a low level of storage considering the amount ingested.

An experiment was carried out to test the speed with which the vitamin A stored in the guinea pig's liver disappeared when the dietary intake was low. Twenty-eight guinea pigs bedded on oat hay and raised on a diet of fresh grass and rolled barley were maintained on the stock diet which provided 0.1 to 0.2 mg. carotene a day. These animals were sacrificed at various intervals and the livers and sera analyzed for vitamin A (table 2). The loss of vitamin A from the liver was rela-

tively rapid. In four of the six animals sacrificed after 30 days on the stock diet, the amount of vitamin A present was so low as to be barely detectable. Of the seven animals sacrificed on the thirty-fourth day the livers of three were devoid of the vitamin. The stores remaining at the end of the 36-day period were low. Fluctuations of serum vitamin occurred with a tendency toward a decrease in the females. There were no signs of vitamin A deficiency in any animal, and on this stock diet of low carotene content the young guinea pigs grew satisfactorily.

TABLE 2

Guinea pigs on low-carotene stock diet showing rate of depletion of liver stores of vitamin A which had been built up by feeding fresh grass.

PERIOD ON STOCK DIET	MALES				FEMALES			
	Number of animals	Weight when sacrificed	Average serum vitamin A	Average total liver vitamin A	Number of animals	Weight when sacrificed	Average serum vitamin A	Average total liver vitamin A
days		*gm.*	*µg./100 ml.*	*µg.*		*gm.*	*µg./100 ml.*	*µg.*
1	3	324–333	33	584	..	...	..	..
3	2	360–384	33	466	1	315	33	156
15	3	434–455	20	222	..	...	..	.
30	5	455–582	26	67	1	500	28	0
34	6	500–580	23	26	1	500	17	0
36	4	482–557	33	31	2	417 359	12	9

Vitamin A and carotene during reproduction

Although growth may be maintained on the low carotene stock diet even though storage is negligible, it was not known whether reproduction was affected by these circumstances. Reproduction was chosen as representing a period of physiological stress during which any advantage inherent in the possession of large reserves of vitamin A might be demonstrated. In its natural environment the guinea pig consumes large amounts of green feed providing a super-abundance of carotene. In consequence, the mechanism of forming and storing vitamin A may be wasteful, and this may operate to the disadvantage of the animal on carotene-low diets.

Good reproduction had been obtained in the stock colony on the somewhat low intake of carotene provided by the stock diet without additional vitamin A. In spite of this the incidence of abortion was high in the guinea pigs observed during this study on the low-carotene stock diet as well as on diet G. Abortions in guinea pigs due to vitamin A

deficiency have been reported by Manzi ('35). Hart and Guilbert ('41) found cattle to be similarly affected. Forty female guinea pigs were used in an attempt to study (a) the adequacy of carotene as compared with the vitamin in meeting the requirement during reproduction and (b) the adequacy of the liver stores for meeting this requirement after the feeding of the two forms of the vitamin.

On the supplemented low-carotene stock diet, which provided 0.15 mg. carotene daily, the animals that received an additional 1.0 mg. carotene daily terminated five of nine pregnancies successfully but without a store of vitamin A in the liver. Three of the four pregnancies supported by the addition of 0.1 mg. vitamin A daily were terminated successfully with significant storage of vitamin A in the liver of both mothers and young.

After a preliminary period on stock diet with the males, during which they received 1.0 mg. carotene or 0.15 mg. vitamin A daily twenty-seven mature young female guinea pigs were placed on the vitamin A deficient diet G. All six of the animals on this diet without added vitamin A died within 8 to 31 days. Of the ten animals that received 0.5 mg. carotene per day seven were pregnant, four of which aborted, and none delivered viable young. Only one survived the experimental period and none had measurable amounts of vitamin A in the liver. Of the eleven animals that received 0.15 mg. vitamin A per day five survived. Four were pregnant and of these two aborted. Living young were not delivered although nearly all of the animals had stores of vitamin A in the liver. The results are suggestive of the superior value of vitamin A over carotene for reproduction but further work must be done before this can be considered established.

SUMMARY

Simplified diets were devised, which when supplemented with lemon juice, ascorbic acid, and vitamin A or carotene were adequate for the growth of guinea pigs. These diets were used to produce vitamin A deficiency when vitamin A and carotene were omitted.

The symptoms of vitamin A deficiency observed in the guinea pig were similar to those described in other species. These were typical xerophthalmia, weight loss, and death within a few days after the eye symptoms developed, usually 3 to 4 weeks after the young animals were placed on the deficient diet.

Storage of vitamin A appeared to be restricted to the liver except for a trace in the kidneys. The liver stores were small except when the preformed vitamin was fed. Vitamin A appeared to be more than six

times as effective for this purpose as was carotene. Daily amounts of 1.0 mg. carotene per kilogram for 38 days in depleted animals produced only a trace of liver vitamin A, whereas 2.0 mg. per kilogram daily for the same period resulted in storage that was just significant. The loss of these stores of the vitamin was rapid on lowered intake, 90% disappearing in 30 days.

LITERATURE CITED

BOOCK, E., AND J. TREVAN 1922 The food value of mangolds and the effects of deficiency of vitamin A on guinea pigs. Biochem. J., vol. 16, p. 780.

CHEVALLIER, A., AND Y. CHORON 1935 Recherches sur la presence de la vitamine A et de la substance A′ dans le tissu hepatique. C. R. Soc. Biol., vol. 118, p. 159.

——— 1936 Sur la mise en reserve de la vitamine A et de la substance A′ dans le tissu hepatique. C. R. Soc. Biol., vol. 121, p. 1015.

CLAUSEN, S. W., AND A. B. MCCOORD 1934 The distribution of vitamin A in the tissues of the rat and guinea pig. J. Biol. Chem., vol. 105, Proc. XV — (XVI).

DAVIES, A. W. 1933 Colorimetric determination of vitamin A by the alkali digestion method. Biochem. J., vol. 27, p. 1770.

GUILBERT, H. R., C. E. HOWELL AND G. H. HART 1940 Minimum vitamin A and carotene requirements of mammalian species. J. Nutrition, vol. 19, p. 91.

HART, G. H., AND H. R. GUILBERT 1941 The relation of brucella abortus infection to the vitamin A content of fetal liver. Am. J. Vet. Res., vol. 2, p. 390.

HETLER, R. A. 1934 The development of xerophthalmia and keratinization of epithelial tissue on withdrawal of vitamin A from the diet of the monkey (Macacus rhesus), guinea pig, rabbit and adult albino rat. J. Nutrition, vol. 8, p. 75.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. J. Biol. Chem., vol. 132, p. 527.

MANZI, D. L. 1925 Valore del carotene nella carenza di vitamin A durante lo stato gravidico. Arch. Ostet. Ginecol., vol. 42, p. 77.

WOLBACH, S. B., AND P. R. HOWE 1928 Vitamin A deficiency in the guinea pig. Arch. Path. and Lab. Med., vol. 5, p. 239.

WOOLLEY, D. W. 1942 Some new dietary essentials required by guinea pigs. J. Biol. Chem., vol. 143, p. 679.

YUDKIN, S. 1941 Estimation of vitamin A and carotene in human blood. Biochem. J., vol. 35, p. 551.

NUTRITIONAL STUDIES ON MILK FAT

I. THE GROWTH OF YOUNG RATS FED MILK FAT AND CERTAIN SYNTHETIC GLYCERIDES AS SUPPLEMENTS TO A FAT-FREE DIET

J. L. HENDERSON, E. L. JACK, SAMUEL LEPKOVSKY AND DELLA F. REID
Division of Dairy Industry, University of California, Davis and Division of Poultry Husbandry, University of California, Berkeley

TWO FIGURES

(Received for publication May 9, 1945)

Until lately, the study of the nutritional value of fats has been complicated by the necessity of using basal diets that contain appreciable quantities of fat supplied in vitamin concentrates. Now, however, one can prepare an adequate basal diet that is essentially fat-free. Such a diet has been compounded and used to compare the growth of rats fed milk fat with the growth of those receiving certain synthetic glycerides as supplements to the basal diet.

EXPERIMENTAL

Test diets

The composition of the fat-free diet (diet 1) is given in table 1. The diets containing fat were made up to contain 20% fat, 30% casein, 46% glucose, 4% salt mixture, and the same vitamin supplements as the fat-free diet. The fats used were trilaurin (diet 2), triolein (diet 3), equal parts trilaurin and triolein (diet 4), and milk fat (diet 5). Triolein was used because it represents the most widely distributed unsaturated fatty acid. Trilaurin was chosen as a saturated fat that melts near body temperature.

The fat-free diet (no. 1), in preliminary trials, gave satisfactory growth when fed to young rats. The only sources of lipids in this diet besides the residual fat in the casein, are the linoleic acid and the ethyl laurate; the latter was used to carry the fat-soluble vitamins, which were dissolved in an amount of ethyl laurate such that about 0.07 ml. per week supplied the required amount to each rat. To ensure consumption of these vitamins and the linoleic acid, they were fed separate from the rest of the diet. The casein was extracted with alcohol

and ether to a lipid content of 0.2%. The original diet was somewhat dusty and this was corrected by substituting 1% glycerol for 1% glucose.

The milk fat was obtained from the milk of a large number of cows; the method of collection has been described elsewhere (Henderson and Jack, '44). The fatty-acid composition, determined in detail (Jack and Henderson, '45) by ester-fractionation and analysis, is shown in table 2.

TABLE 1

Composition of the fat-free diet (diet 1).

Basic mixture		*Other vitamin supplements fed per kilo of basic mixture*	
Labco casein, alcohol and ether extracted	24%	Thiamine [3]	10 mg.
Dextrose	72%	Riboflavin [3]	10 mg.
Sure's salt mixture [1]	4%	Pyridoxine [3]	10 mg.
Vitamin supplements		Pantothenic acid [3] (as Ca-pantothenate)	40 mg.
Linoleic acid, prepared by fractional crystallization in acetone (−60°C.) of fatty acids of corn oil	25 mg./rat/day	Nicotinic acid	0.1 gm.
α-Tocopherol (Eastman)	2 mg./rat/wk.	Inositol	1.0 gm.
Vitamin A alcohol (Eastman)	30 µg./rat/wk.	Para-aminobenzoic acid	0.6 gm.
Vitamin D_3 [2]	1 µg./rat/wk.	Choline	500 mg.
Vitamin K (1-acetoxy-2 methyl-4-naphthyl sodium phosphate)	2 µg./rat/wk.	Biotin [4]	25 µg.

[1] Sure, B., J. Nutrition, vol. 22, p. 499, 1941.

[2] Furnished by courtesy of E. I. du Pont de Nemours and Co., New Brunswick, N. J.

[3] Supplied by courtesy of Merck and Co., Rahway, N. J.

[4] Biotin solution (free acid), S.M.A. Corp., Chagrin Falls, Ohio.

TABLE 2

Fatty-acid composition of milk fat.

(Iodine no. (Hanus) 32.42; saponification no. 230.0; nonsaponifiable matter 0.28).

LENGTH OF CARBON CHAIN	WEIGHT	MOL	LENGTH OF CARBON CHAIN	WEIGHT	MOL
	%	%		%	%
Saturated			Unsaturated		
C_4	3.52	9.2	C_{10}	0.25	0.3
C_6	1.40	2.8	C_{12}	0.15	0.2
C_8	1.68	2.7	C_{14}	1.48	1.5
C_{10}	2.67	3.5	C_{16}	5.69	5.2
C_{12}	4.54	5.2	C_{18}	18.69	15.3
C_{14}	14.65	14.8	C_{20}	0.98	0.7
C_{16}	30.05	27.2	Linoleic	2.12	1.7
C_{18}	10.45	8.5			
C_{20}	1.68	1.2			

Oleic acid for the triolein was prepared from olive oil by the fractional-crystallization method of Brown and Shinowara ('37). The lauric acid for the trilaurin was obtained by ester fractionation of coconut oil. To prepare the triglycerides, direct esterification of the acids with glycerol under bubbling CO_2 was the method used. The esterified products were subsequently washed with 70% alcohol.

The test diets were mixed in small quantities and refrigerated to avoid deterioration.

Feeding trials

Two trials were made using young rats from the stock colony divided into groups of four each, two males and two females. They were maintained in individual cages and fed the diets ad libitum. Over-all feed consumption was measured. In one trial (no. 1) the mothers and young were placed on the fat-free diet when the young were 14 days old. The young rats were weaned at 21 days and placed on the test diets at an average weight of about 48 gm. In the other trial (no. 2) the rats were allowed to grow to about 85 gm. before weaning; they were then placed on the fat-free diet for 1 week before being given the test diets.

DISCUSSION

The results of the two feeding trials are illustrated in figures 1 and 2. Mackenzie, Mackenzie and McCollum ('39) prepared a diet lower in fat than any previously reported. It was found to give growth comparable with that made on their stock diet. The fat-free diet used in the present experiments was planned along the same lines as the Mackenzie-McCollum study except that glucose was used in place of sucrose, the B vitamins were provided as pure materials rather than from extracted yeast, and no cystine was included. The growth rates of our rats on fat-free diets closely resembled those obtained by Mackenzie et al. ('39).

The difference in growth rates between the two experiments here reported can be explained by the difference in starting weights. The smaller rats grow more rapidly than the larger ones; but if the curves are superimposed so that the ordinates coincide at the same body weight, they form segments of the same general curve.

It is difficult to ascribe any significance to the fat-depletion period, either of the mother before weaning or of the young rats after weaning. In both trials, rats receiving the milk fat and the triolein showed faster growth rates than those receiving the other diets. There was no significant difference between the milk fat and the triolein diets.

Trilaurin restricted growth as has been reported earlier in the literature (Evans and Lepkovsky, '32 a, '32 b; Cox, '33) and the effect was more pronounced in the younger rats. When trilaurin was mixed with triolein so that each provided 10% of the diet, this diet was less restrictive of growth than when the diet contained 20% trilaurin.

Feed consumption was almost identical for all lots, except that those on the trilaurin ate slightly less feed than the others. This difference, however, being only about 5% less feed per rat for the entire feeding period, was too small to account for the difference in growth.

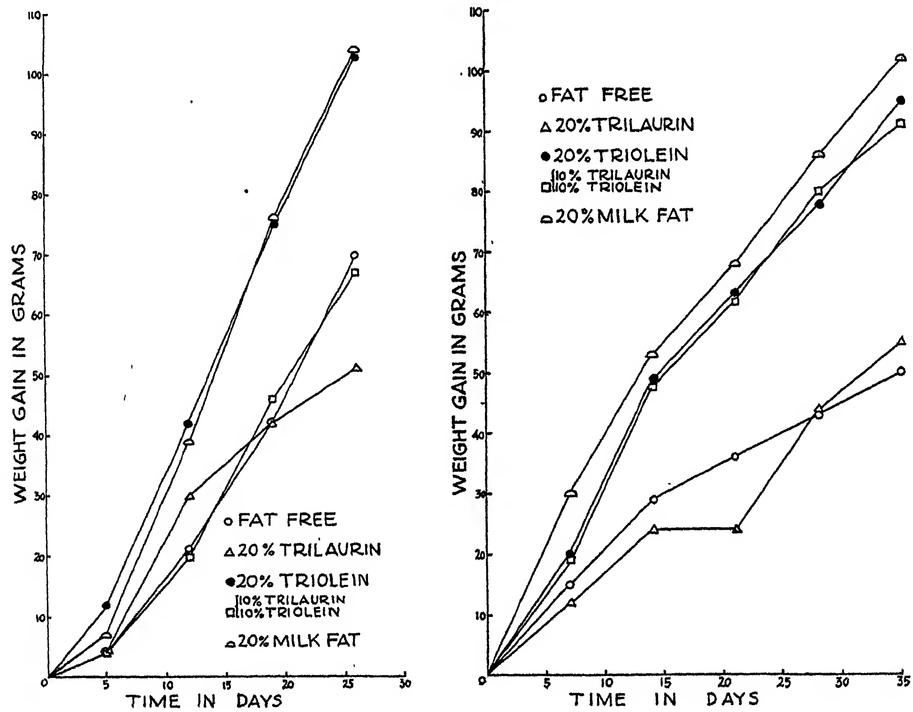


Fig. 1 Weight gain of rats in feeding trial 1.
Fig. 2 Weight gain of rats in feeding trial 2.

SUMMARY AND CONCLUSION

1. A fat-free diet is described in which all the components are pure compounds with the exception of casein extracted with alcohol and ether.

2. This diet was found to be satisfactory for use in the fat-nutritional experiments.

3. The rats on the milk-fat diet and on the triolein diet grew faster than those on the other diets. There was no significant difference be-

tween the milk-fat diet and the triolein diet with respect to their effect on growth.

4. Trilaurin, when fed at the level of 20%, enabled the rats to grow at about the same rate or slightly slower than the rats on a fat-free diet.

5. When 10% each of trilaurin and triolein were fed, the growth of the rats was intermediate between those receiving trilaurin and those receiving triolein.

6. The growth-promoting effects of the different diets could not be attributed to differences in feed consumption.

LITERATURE CITED

BROWN, J. B., AND G. Y. SHINOWARA 1937 Studies on the chemistry of the fatty acids. II. The preparation of pure oleic acid by a simplified method. Am. Chem. Soc. J., vol. 59, p. 6.

COX, W. M. 1933 The nutritive value of pure fatty acid esters. J. Biol. Chem., vol. 103, p. 777.

EVANS, H. M., AND S. LEPKOVSKY 1932 a Vital need of the body for certain unsaturated fatty acids. II. Experiments with high fat diets in which saturated fatty acids furnish the sole source of energy. J. Biol. Chem., vol. 96, p. 157.

——— 1932 b The sparing action of fat on vitamin B. III. The role played by glycerides of single fatty acids. J. Biol. Chem., vol. 96, p. 179.

HENDERSON, J. L., AND E. L. JACK 1944 The fractionation of milk fat from a solvent at low temperatures. Oil and Soap, vol. 23, p. 90.

JACK, E. L., AND J. L. HENDERSON 1945 The fatty-acid composition of glyceride fractions separated from milk fat. J. Dairy Sci., vol. 28, p. 65.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLOM 1939 Growth and reproduction on a low fat diet. Biochem. J., vol. 33, p. 935.

NUTRITIONAL STUDIES ON MILK FAT

II. THE GROWTH OF YOUNG RATS FED GLYCERIDE FRACTIONS SEPARATED FROM MILK FAT

E. L. JACK, J. L. HENDERSON, DELLA F. REID AND SAMUEL LEPKOVSKY
Division of Dairy Industry, University of California, Davis and Division of Poultry Husbandry, University of California, Berkeley

ONE FIGURE

(Received for publication May 9, 1945)

The opinion is current in many places that food fats of either animal or vegetable origin, when properly supplemented, are practically equivalent in nutritional value. The Council on Foods and Nutrition of the American Medical Association ('42) drew the following conclusion: "At present there is no scientific evidence to show that the use of fortified oleomargarine in the average adult diet would lead to nutritional difficulties. A similar conclusion is probably justified in the case of growing children." This statement has been taken by many persons to imply that oleomargarine is nutritionally equal to milk fat. The Council, however, further points out: "The nutritional factors have not all been identified and butter contains numerous additional fatty acids of unknown nutritional significance."

Deuel and associates ('44) were able to find no difference in the growth rate of rats fed diets supplemented with butter, corn oil, cottonseed oil, margarine, olive oil, peanut oil, and soybean oil. The fats were fed at approximately a 30% level. On the other hand Gullickson, Fountaine and Fitch ('42) found that young calves did poorly and often died when their sole source of fat was vegetable oils, but that they were healthy and thrifty on milk fat, lard, or tallow. Elvehjem, Hart, and their co-workers ('40, '40, '41) showed that milk fat gave better growth when fed to weanling rats than did corn oil, coconut oil, cottonseed oil, and soybean oil. The superiority of milk fat was most pronounced when lactose was the carbohydrate used (Boutwell et al., '43). The corn oil was superior when dextrin, sucrose, glucose, or starch was used. According to these results and others reported in the literature, there is no clear-cut evidence that one fat is nutritionally superior to another.

The present paper reports the results obtained when glyceride fractions from a natural fat (milk fat) were fed to weanling rats as supplements to a fat-free diet. This approach to the problem was chosen because if any portions of a natural fat (whose composition is known) can be shown to differ nutritionally from other portions, then a basis will be established for determining the inherent nutritional value of a fat.

EXPERIMENTAL

Fat fractions

Milk-fat fractions were prepared by precipitation from a solvent at progressively lower temperatures, as follows: — 7°, — 13°, — 23°, — 53°C., and the filtrate remaining at — 53°C. The details of the preparation of these fractions have been described elsewhere (Henderson and Jack, '44). In physical appearance these fractions ranged from a dry white powder to a reddish oil; the melting point ranged from 53° to — 10°C., and the iodine numbers from 8.2 to 56. The detailed fatty-acid composition of the fractions and of the original milk fat is shown in table 1 (Jack and Henderson, '45).

TABLE 1

Fatty acid composition of milk fat and of milk-fat fractions.

LENGTH OF CARBON CHAIN	MOL PER CENT					
	— 7 ppt.	— 13 ppt.	— 23 ppt.	— 53 ppt.	— 53 filt.	Milk fat
Saturated						
C_4	2.5	4.6	8.7	7.9	9.3	9.2
C_6	..	2.9	4.0	5.8	6.9	2.8
C_8	..	6.6	1.3	1.7	2.4	2.7
C_{10}	1.2	4.9	4.0	5.5	4.5	3.5
C_{12}	4.5	3.5	4.2	4.7	3.7	5.2
C_{14}	16.6	11.3	14.6	16.6	7.4	14.8
C_{16}	42.1	36.2	38.1	24.0	14.0	27.2
C_{18}	21.1	9.3	5.2	8.5	6.8	8.5
C_{20}	3.8	3.1	1.0	1.4	1.4	1.2
C_{22}	0.8	..	..	..	.	..
Unsaturated						
C_{10}	.	0.2	0.1	0.3	1.4	0.3
C_{12}	..	0.2	0.1	0.2	0.4	0.2
C_{14}	0.1	0.9	1.2	1.4	1.8	1.5
C_{16}	0.4	3.9	4.5	2.7	4.6	5.2
C_{18}	6.1	10.6	10.7	17.2	30.0	15.3
C_{20}	0.8	1.3	1.9	1.8	0.8	0.7
Linoleic	..	0.5	0.4	0.5	6.0	1.7

Test diets

Table 2 shows the compositions of the diets.

TABLE 2

Composition of test diets.[1]

DIET NO.	FAT SOURCE AT 20% LEVEL	CASEIN	GLUCOSE	SURE'S SALT MIX
		%	%	%
1	Fat-free	24	72	4
2	Milk fat (solvent treated)	30	46	4
3	Synthetic[2] milk fat	30	46	4
4	— 7 ppt.	30	46	4
5	— 13 ppt.	30	46	4
6	— 23 ppt.	30	46	4
7	— 53 ppt.	30	46	4
8	— 53 filt.	30	46	4
9	Composite[3] milk fat	30	46	4
10	Milk fat (not solvent treated)	30	46	4

[1] The diets were supplemented by vitamins, as described previously. See table 1 in paper entitled "Nutritional Studies on Milk Fat. I." by Henderson and Jack, vol. 30, p. 170.

[2] Fatty acids of milk fat reesterified with glycerol.

[3] Combined increments of the different fractions.

Feeding technic

The selection of the rats, the numbers used, and the methods of feeding were the same as those previously described (Henderson et al., '45).

RESULTS

Two feeding trials were made, and the results of trial 2 are shown in figure 1 as being representative. The findings were practically identical in the two trials. Trial 2 was chosen for presentation because it included diets 2 and 3 which were not in the first trial. Diet 2 contained milk fat which had been dissolved in solvent followed by solvent removal in the same manner as the fat fractions. The fat in diet 3 was synthesized from the component fatty acids of milk fat.

DISCUSSION

The experimental data illustrated in figure 1 show the diet containing untreated milk fat to be superior to the others. The remainder of

the diets, all of which contained fat which had been dissolved in solvent at some stage in its preparation, may be divided essentially into three groups on the basis of their growth-promoting ability: the diet containing the — 53° filtrate was markedly superior to the rest; the diets containing the — 7° ppt. and the fat-free diet were the poorest; the others, while showing some variation within themselves, were all sufficiently in the same range to constitute a single group. The authors are not prepared to attempt an explanation of the effect of the solvent.

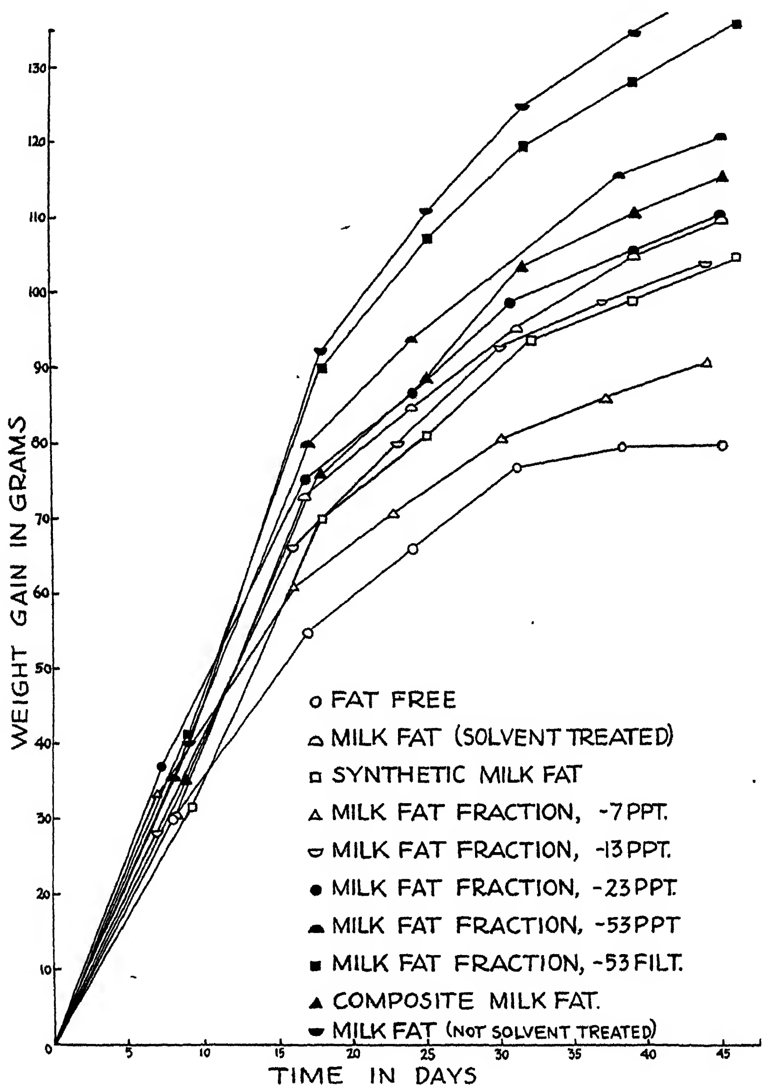


Fig. 1 Weight gain of rats on diets containing milk-fat derivatives.

The diets containing the fat fractions, even though the two extreme fractions are outstanding, all ranged themselves in the order of the temperature of precipitation. These differences cannot be ascribed to differences in assimilation resulting from differences in melting point (Henderson and Jack, '44), since all except the — 7° ppt. melt at temperatures at which assimilation is known to be excellent, and since even 53° C., the melting point of the — 7° ppt., was not high enough to interfere seriously with assimilation (Boutwell et al., '43).

Reference to the composition of the fat fractions (table 1) suggests that the oleic acid content or possibly the total unsaturation might be a contributing factor since this is the only characteristic that varies in the same manner as the growth-promoting ability. Conceivably, the marked superiority of the — 53° filtrate might also result from the fact that this fraction is highly unsaturated and contains most of the nonsaponifiable matter of milk fat. This possibility, however, does not appear probable, since preliminary trials have shown the fat-free diet to be vitamin-sufficient; it is believed that the principal role of these factors is to supply vitamin activity.

The results presented here suggest conclusions different from those of the Wisconsin workers (Schantz et al., '40), wherein they reported that the saturated portion of milk fat provided better growth than the other portions. Their saturated portion compared in degree of unsaturation with our — 7° ppt., which was inferior to the other fractions. The results here were on the natural glycerides, whereas the Wisconsin results were based on glycerides synthesized from "solid" fatty acids separated from milk fat. The results obtained by the Wisconsin feeding technic could be interpreted as showing the value of saturated fatty acids of milk as supplements to corn oil, since the fatty-acid fractions fed had been mixed with and replaced a certain amount of corn oil. Possibly the differences were due to the synergistic action of the two fats, or fat-soluble constituents, rather than to an inherent property of either. In the results here reported, the differences are believed to be true representations of the growth-promoting properties of milk-fat fractions, inasmuch as they constituted the sole source of fat.

Essentially similar results were obtained with the natural milk fat, treated with the same solvent as the fractions; the synthetic milk fat, prepared by esterification of the milk-fat fatty acids with glycerol; and the composite milk fat, obtained by mixing the proper increments of the milk-fat fractions. The results fell about midway in the intermediate group. The synthetic milk fat does not contain the nonsaponifiable matter of milk fat, which is present in the other two.

Burr and Barnes ('43) have commented on the large differences that have been reported on the comparative nutritional value of different lipids. According to them, the outstanding feature of these experiments is the irregularity of results, which they ascribe either to the method of feeding or to the unrecognized effect of rancidity on the rest of the diet. We do not believe that either of these two factors affected our findings, since the experiments were repeated after 5 months with the same results, using fat from the same source.

SUMMARY AND CONCLUSION

1. Five glyceride fractions separated from milk fat by precipitation from a solvent were fed to weanling rats, and the gain in weight was measured.

2. The growth responses place the fractions in three groups. The — 53° filtrate was superior to all other diets containing solvent-treated fat; the — 7° ppt. and the fat-free were the poorest; the others formed an intermediate group.

3. The diet containing natural milk fat which had not been solvent-treated gave greater growth than any other diet. The diets containing the solvent-treated milk fats (natural, synthetic and composite) fell in the intermediate group with no significant difference between them.

4. The fatty acid composition of the fats suggests that either the oleic acid content or the total unsaturation might be a factor contributing to differences in growth.

LITERATURE CITED

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1941 The effect of hydrogenation on the nutritive value of the fatty acid fractions of butter fat and of certain vegetable oils. J. Dairy Sci., vol. 24, p. 1027.

——— 1943 Further studies on the growth promoting value of butter fat. J. Dairy Sci., vol. 26, p. 429.

BURR, G. O., AND R. H. BARNES 1943 The non-caloric value of dietary fats. Physiol. Rev., vol. 23, p. 256.

COUNCIL ON FOODS AND NUTRITION OF THE AMERICAN MEDICAL ASSOCIATION 1942 The comparative nutritional value of butter and oleomargarine. J. Am. Med. Assn., vol. 119, p. 1425.

DEUEL, H. J., JR., E. MOVITT, L. F. HALLMAN AND F. MATTSON, WITH TECHNICAL ASSISTANCE OF E. BROWN 1944 Studies on the comparative nutritive value of fats. I. Growth rate and efficiency of conversion of various diets to tissue. J. Nutrition, vol. 27, p. 107.

GULLICKSON, T. W., F. C. FOUNTAINE AND J. B. FITCH 1942 Various oils and fats as substitutes for butterfat in the ration of young calves. J. Dairy Sci., vol. 25, p. 117.

HENDERSON, J. L., AND E. L. JACK 1944 The fractionation of milk fat from a solvent at low temperatures. Oil and Soap, vol. 21, p. 90.

HENDERSON, J. L., E. L. JACK, S. LEPKOVSKY AND D. F. REID 1945 Nutritional studies on milk fat. I. The growth of young rats fed milk fat and certain synthetic glycerides as supplements to a fat-free diet. J. Nutrition, vol. 30, p. 169.

JACK, E. L., AND J. L. HENDERSON 1945 The fatty-acid composition of glyceride fractions separated from milk fat. J. Dairy Sci., vol. 28, p. 65.

SCHANTZ, E. J., R. K. BOUTWELL, C. A. ELVEHJEM AND E. B. HART 1940 The nutritive value of the fatty acid fractions of butter fat. J. Dairy Sci., vol. 23, p. 1205.

SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1940 The comparative nutritive value of butter fat and certain vegetable oils. J. Dairy Sci., vol. 23, p. 181.

ENZYMATIC RELATIONSHIPS IN THE UTILIZATION OF SOYBEAN OIL MEAL PHOSPHORUS BY THE RAT [1]

ROBERT R. SPITZER AND PAUL H. PHILLIPS
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

(Received for publication May 31, 1945)

INTRODUCTION

In a study of the availability of soybean oil meal phosphorus (Spitzer and Phillips, '45) it was found that 58.0% of the soybean oil meal phosphorus existed in the form of phytin or phytic acid and that this phosphorus was readily available to the rat.

In view of these results an explanation for the utilization of soybean oil meal phosphorus was sought by investigating the role of phytin and phytic acid-splitting enzymes.

A phytic acid-splitting enzyme (phytase) was first found by Suzuki et al. ('06). Adler ('16) studied a malt phytase and found its optimum hydrogen ion concentration at pH 5.4. Horiuchi ('31) found that rice bran contained a phytase with optimum hydrogen ion concentration at pH 4.3.

It has been suggested by Hart et al. ('09), by Pedersen ('41) and by Singsen and Mitchell ('44) that there may be some relationship between the availability of phytic acid phosphorus and the phytase activity of the ration. McCance and Widdowsen ('44) and Mellanby ('44) have discussed the role of phytase in wheat and oats with respect to the difference in rachitogenic effect between the two grains.

The occurrence of phytase in the animal body was reported by McCollum and Hart ('08). These workers found phytase activity in the liver and blood of calves. Plimmer ('13) failed to find the enzyme in

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This study has been supported in part by a grant from the A. E. Staley Manufacturing Company, Decatur, Illinois.

We are indebted to Merck and Co., Rahway, New Jersey, for the synthetic vitamins; to Wilson and Company, Chicago, Illinois, for the 1: 20 liver powder; to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil, and to Oscar Mayer Packing Company, Madison, Wisconsin for animal tissues used in these studies.

extracts of the intestines of the dog, sheep, ox and rabbit. Lowe and Steenbock ('36) failed to find phytin-phosphatase activity in the extracts of the mucosa of the small intestine of the chick or the rat. These workers suggested that the hydrolysis occurring in the tract was due to the activity of the intestinal flora or to the phytase in the vegetable part of the ration.

Patwardhan ('37) reported phytase activity in the intestine of the albino rat. This enzyme exerted its optimum activity at pH 7.8 and its action was reported to be accelerated by magnesium ions. Kreiger ('38) reported the existence of a phytin-splitting enzyme in the small intestinal wall of the rat and the chick. Magnesium ions when present at the proper concentration had a stimulating effect on phytase activity. The optimum pH range for this enzyme was between pH 6.8 and pH 7.0. Rapoport et al. ('41) found no phytase activity in the cells or plasma of several species of mammals, but phytase was present in the plasma of lower vertebrates. This enzyme showed optimum activity at a slightly acid pH and was not stimulated by magnesium ions.

The earlier studies by Spitzer and Phillips ('45)[2] which suggested that the presence of an enzyme in soybean oil meal was not required for utilization of soybean oil meal phosphorus by the rat did not rule out the possibility that other ration constituents possessed phytase activity.

The purpose of the present experiment was to determine if the availability of the soybean oil meal phosphorus was due to the action of a ration-borne enzyme, or the presence of phytase in the gastrointestinal tract or in the organs themselves. To this end a method for measuring phytase activity was developed, and the phytase activity of several soybean oil meals, rations, and tissues was measured.

EXPERIMENTAL

Studies were made with 40 gm. weanling rats fed a semi-synthetic basal ration which was low in phosphorus to determine if the availability of soybean oil meal phosphorus was due to the presence of phytase in the ration. The basal ration differed from the one used in

[2] The ration used in these studies was similar in composition to the low-P-fibrin ration used by Jones (J. Nut. 15, 269, 1942) in that it contained fibrin as the source of protein and a low phosphorus salt mixture. The publications of Jones and associates have amply demonstrated that their low phosphorus-fibrin rations were adequate for growth and bone formation in the rat when phosphorus was added. The growth rates and bone ash values obtained by Spitzer and Phillips, '45 are in very close agreement to those obtained by Jones and Foster (J. Nut. 24, 245, 1942).

earlier studies in one respect; namely, the 1% 1:20 liver powder was omitted because it was a constituent which might possess some phytase activity. This basal ration which will be called the "basal-B" ration consisted of 77.6% sucrose, 14.0% fibrin, 2% low calcium low phosphorus salts, 4% corn oil, 0.1% inositol and 0.3% choline chloride. The other vitamins were fed at the same level and in the same manner that has been reported earlier (Spitzer and Phillips, '45). Vitamin D was fed at a level of 70 U.S.P. units per week.

The "basal-B" ration contained 0.014% phosphorus as determined by the method of Fiske and Subbarow ('25) and the use of an Evelyn photoelectric colorimeter. The phosphorus content of all rations was determined in order to provide data on the level of this element fed throughout the experiment. The calcium content of the rations in all cases was kept as near as possible to 0.6% supplied either as $CaHPO_4$ or $CaCO_3$ or both. The Ca : P ratio varied within the limits of 2.4 : 1 and 2.8 : 1. Inorganic phosphorus was added as $CaHPO_4$. All major substitutions in the "basal-B" ration were made at the expense of the sucrose or fibrin or both.

Rations were stored in the refrigerator to avoid the development of rancidity. Food and distilled water were supplied daily ad libitum. The rats, six per lot, were kept in cages of galvanized wire.

At the end of 5 weeks the animals were sacrificed, the femora removed and bone ash determined by the technique described in earlier experiments (Spitzer and Phillips, '45). The dietary regimen is given in table 1.

RESULTS

Growth data are summarized in figure 1. Inspection of these data shows that when 1.0% 1:20 liver powder was fed with fibrin or soybean oil meal, better growth resulted than when the fibrin or soybean oil meal was fed alone. Heating either the solvent or the expeller type soybean oil meal portion of the ration at 98°C. for 48 hours had no detrimental effect upon growth. When, however, the entire rations were heated at 98°C. for 48 hours, a marked reduction in food consumption occurred which resulted in a retardation of growth rate.

Table 1 summarizes the data on the availability of soybean oil meal phosphorus as measured by bone ash. It is evident that heating the soybean oil meals or the entire ration had no unfavorable effect on the availability of the phosphorus. Neither did the removal of the 1.0% 1:20 liver powder. Slightly higher bone ash values were obtained when 1.0% of 1:20 liver powder was present but this small increase may be

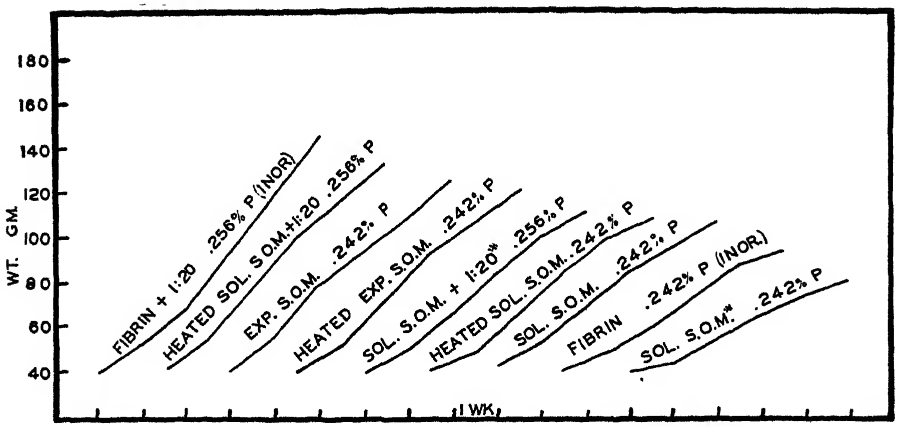


Fig. 1 The effect of heat and the removal of 1.0% liver powder on growth when certain soybean oil meal and fibrin rations were fed. The entire ration was heated for 48 hours at 98°C.

TABLE 1

The effect of heat on the availability of soybean oil meal phosphorus.

LOT NO.	NO. OF RATS	RATION	PER CENT PHOSPHORUS IN RATION	PER CENT CALCIUM IN RATION	AVERAGE PER CENT BONE ASH
1	6	Basal B without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	0.242	0.69	54.2
2	6	Basal B without fibrin + 34% expeller soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	0.242	0.69	54.1
3	6	Basal B without fibrin + 34% solvent soybean oil meal (not toasted, 14% protein and 0.228% P)	0.242	0.69	52.0
4	6	Basal B without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	0.242	0.69	52.0
5	6	Basal B without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P) + 1% 1: 20 liver powder	0.256	0.69	53.2
6	6	Basal B without fibrin + 34% solvent soybean oil meal (14% protein and 0.228% P). Entire ration heated 48 hours, 98°C.	0.242	0.69	50.5
7	6	Basal B without fibrin + 34% solvent soybean oil meal (14% protein and 0.228% P) + 1% 1: 20 liver powder. Entire ration heated 48 hours, 98°C.	0.256	0.69	51.0
8	6	Basal B + 0.228% inorganic P	0.242	0.60	47.1
9	6	Basal B + 1% 1: 20 liver powder + 0.228% inorganic P	0.256	0.60	48.5

explained on the basis of the phosphorus present in the 1:20 liver powder itself. This slight increase was also observed on the fibrin rations where inorganic phosphorus was employed. It is interesting to observe that although heating the ration caused a depression in growth (fig. 1) and in food consumed, it had very little or no effect on bone ash value.

These results indicate that the utilization of soybean oil meal phosphorus was not dependent upon a ration-borne heat labile phytase.

Since Patwardhan ('37) and Kreiger ('38) had reported phytase activity in the intestine of the rat, it seemed possible that this was the source of the enzyme responsible for the utilization of the soybean oil meal phosphorus. Consequently a method for the measurement of phytase activity was developed for the purpose of ascertaining its distribution in tissues and to check the phytase activity of our rations.

Determination of phytase

Preparation of substrate. Calcium phytate was dissolved in dilute hydrochloric acid and the solution filtered. An equivalent amount of sodium oxalate was then added and the solution was made slightly alkaline. The precipitate of calcium oxalate was removed by filtration. The filtrate containing the sodium phytate was analyzed for inorganic and total phosphorus by the method of Fiske and Subbarow ('25). The results showed the presence of 100 μg. of sodium phytate phosphorus and 10.0 μg. of inorganic phosphorus per milliliter.

Preparation of samples. Albino rats were decapitated, the small intestines were removed, opened, washed carefully in running water, and homogenized by the Potter-Elvehjem technique ('36). Large animal intestines were obtained immediately after the animals were slaughtered and were treated in the manner described above. Thus samples of intestinal walls were prepared for analyses. Samples of intestinal contents were extracted with distilled H_2O and the insoluble material removed by filtration. Plant tissues were prepared by making a water extract of a finely ground sample.

Method of assay. To 2.0 ml. of substrate and 3.0 ml. of buffer, 1.0 ml. of the tissue preparation was added. Inorganic phosphorus was determined immediately before and after a suitable incubation period at 37°C. The increase in inorganic phosphorus represented the phytase activity when other mechanisms for the release of phosphorus were accounted for. The proteins were precipitated by the addition of 3 ml. of 10.0% trichloracetic acid. After standing for 10 minutes the material

was filtered and a suitable aliquot used for the phosphorus determination. The increase in inorganic phosphorus due to the action of other phosphatases and to the breakdown of other phosphorus compounds present in the tissue was controlled by replacing the sodium phytate substrate with distilled water and measuring the liberated phosphorus after incubation. Omission of the tissues from the media resulted in no increase in inorganic phosphorus after an incubation period of 6 hours, which indicated that the substrate could not be hydrolyzed by acid or base alone in the absence of phytase. Thus the increase in inorganic phophorus, due to the action of phytase on the sodium phytate was obtained by difference.

Results of phytase experiments

Preliminary phytase determinations using the above method indicated the presence of the enzyme in the mucosa of the small intestine of the rat. Further analyses were made to study the effect of time, of hydrogen ion concentration, and of magnesium ions on the enzyme's action. Other tissues were also analyzed for the enzyme.

The effect of time on the hydrolysis of sodium phytate by rat intestine. An incubation period of 6 hours was chosen since incubation for this period of time resulted in a readily measurable increase in inorganic phosphorus if phytase was present. Tissues showing no phytase activity after 6 hours also failed to show activity after a 48-hour incubation period.

The effect of hydrogen ion concentration on the hydrolysis of sodium phytate by rat intestine. These results are summarized in figure 2. Potassium acid pthalate buffers were used to give pH 2.2, 3.0, 4.0, 5.2 and 6.0 A sodium acetate buffer was used at pH 7.0 and borate buffers were used at pH 7.8, 9.0 and 10.0. The original media contained approximately 200 µg. of sodium phytate phosphorus. Maximum activity was observed in the region of pH 7.8 where 197 µg. or approximately 98% of the phytate phosphorus was liberated during the 6-hour incubation period.

The influence of magnesium ions on the hydrolysis of sodium phytate by rat intestine. Duplicate determinations were carried out simultaneously to study the effect of magnesium ions on the action of phytase. The media was buffered at pH 7.8 and a 6-hour incubation period was used. The results of the studies are outlined in table 2. Although the addition of a few crystals of $MgSO_4$ to the media resulted in an increase in total phosphorus liberated, it had no stimulating effect on the phytase activity at this pH.

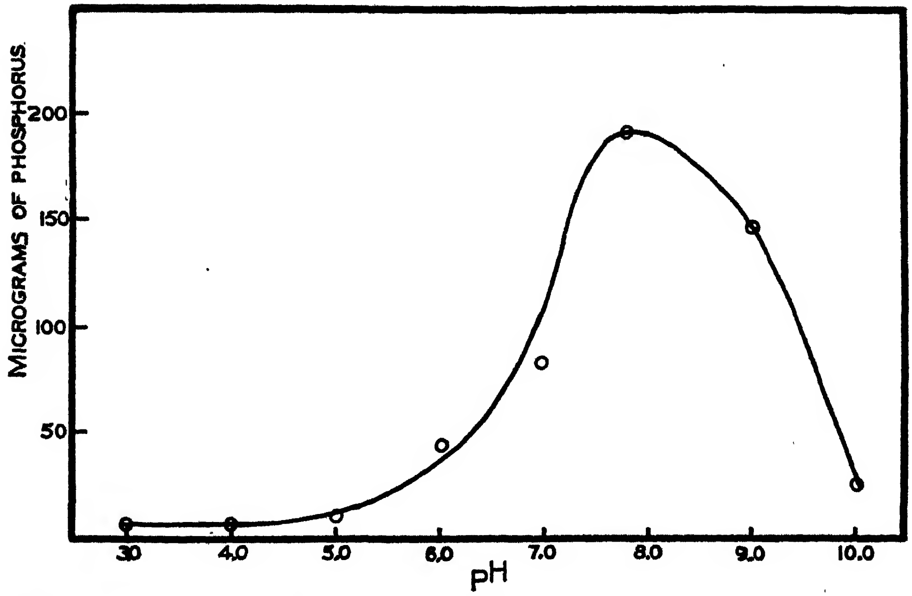


Fig. 2 The effect of hydrogen ion concentration on the hydrolysis of sodium phytate by rat intestine.

TABLE 2

The effect of magnesium ions on the total phosphatase and phytase activity of rat intestine.

	Added $MgSO_4$	Incubation time in hours	Micrograms phosphorus liberated	Micrograms Na phytate phosphorus liberated
Enzyme preparation, no. 1				
With substrate	0	0		
With substrate	0	6	237	
Without substrate	0	0		
Without substrate	0	6	73	164
With substrate	+	0		
With substrate	+	6	246	
Without substrate	+	0		
Without substrate	+	6	103	143
Enzyme preparation, no. 2				
With substrate	0	0		
With substrate	0	6	224	
Without substrate	0	0		
Without substrate	0	6	72	152
With substrate	+	0		
With substrate	+	6	252	
Without substrate	+	0		
Without substrate	+	6	109	143

The effect of ration ingested upon the phytase activity of rat intestine and rat intestinal contents. The small intestines of the rats fed rations used in the previous studies and animals of varying ages receiving a stock ration [3] were used in these studies. All animals, twenty-eight in number, showed phytase activity in the mucosa of the small intestine. Some phytase activity was observed in the intestinal contents.

The distribution of phytase in the tissues of other animals. Extension of these studies to other species has shown phytase to be present in the small intestinal mucosa of the chick, pig and cow. Some phytase activity was also observed in the intestinal contents of the cow and the pig. Determinations were made at pH 7.8 on tissue samples from three different animals of each species.

The distribution of phytase in rations used. Analysis failed to show any phytase activity at either pH 7.8 or 5.2 in solvent or extracted soybean oil meals, or in the rations before or after heat treatment.

DISCUSSION

The crude enzyme preparations used in these studies undoubtedly contained some magnesium ions. Further additions of magnesium ions resulted in an increase in total inorganic phosphorus liberated but had no stimulating effect on the phytase. Kay ('32) reported that an intestinal phosphatase, capable of hydrolyzing monophosphoric esters, was stimulated by magnesium ions. However, the phytase reported by Rapoport et al. ('41) showed its maximum activity at a slightly acid pH and was not stimulated by magnesium ions. Similar to our studies, Rapoport used methods for determining the increase in inorganic acid phosphorus due specifically to phytase. This suggests that the stimulation observed by Patwardhan ('37) and Kreiger ('38) was due to the activation of other phosphatases, as shown by Kay ('32). The question of concentration of magnesium ions should not be overlooked.

It seems quite possible that the enzyme studied here was the same phytase reported by Patwardhan ('37) and possibly similar to that reported by Kreiger ('38). On the other hand the existence of several animal "phytases" is quite possible.

Kreiger and Steenbock ('40) have shown that the utilization of phytin phosphorus by the rat is enhanced by feeding high levels of vitamin D and by employing a favorable Ca : P ratio. In our studies in which we have shown utilization of soybean oil meal phosphorus (58%

[3] Stock ration consisted of wheat, corn, linseed oil meal, soybean oil meal, tankage, ground alfalfa, butter, liver powder, $CaCO_3$ and NaCl.

in the form of phytin or phytic acid), 70 U.S.P. units of vitamin D were fed weekly and favorable Ca: P ratios were maintained.

Extension of these studies to other species has demonstrated phytase in the mucosa of the small intestine of the chicken, pig and cow. Some activity was also observed in the intestinal contents of the pig and cow. Under practical conditions where sufficient vitamin D is obtained from solar irradiation it seems likely that these animals too would utilize phytin or phytic acid phosphorus since phytase is present in their intestinal mucosas. This would explain the results obtained by Hart et al. ('09) and Singsen and Mitchell ('44). According to Kreiger ('38) it seems quite possible that vitamin D is directly related to the activity of phytase.

No phytase activity could be measured in the rations used in these studies. The importance of plant phytases in the utilization of phytin and phytic acid phosphorus has been discussed by McCance and Widdowsen ('44), Mellanby ('44), Pedersen ('41) and Singsen and Mitchell ('44). Plant phytases that have been reported show optimum activity under slightly acid conditions. These may function in hydrolyzing phytin and phytic acid during certain cooking procedures or in the acid parts of the digestive tract. However, the importance of the plant phytases is probably over-emphasized.

The possible phytase activity of the intestinal flora should not be overlooked since the intestinal contents of the rat, pig and cow exhibited some activity.

SUMMARY AND CONCLUSIONS

It has been shown that soybean oil meal phosphorus, which was 58% phytin or phytic acid phosphorus, was utilized by the rat without a ration-borne heat labile phytase. Heating the rations at 98°C. for 48 hours did not depress the utilization of the phosphorus.

A method for the determination of phytase activity was developed which revealed the presence of a phytase in the small intestinal mucosa and in the intestinal contents of the rat. The optimum pH of this enzyme was found to be pH 7.8. Although the addition of magnesium ions to a crude enzyme preparation caused an increase in total phosphorus liberated, it did not stimulate the action of phytase. Phytase activity was observed in the small intestines of rats of all ages and in the small intestines of rats receiving several different rations.

Solvent or expeller soybean meals failed to show any phytase activity at pH 5.2 or at pH 7.8.

Phytase activity was observed in the small intestinal mucosa of the chicken, pig and cow.

From these studies it would appear that the phytin or phytic acid phosphorus of soybean oil meal was made available by the action of intestinal phytases.

LITERATURE CITED

ADLER, L. 1916 Gewinnung von Phytase aus Malz. Biochem. Z., vol. 75, p. 319.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

HART, E. B., E. V. MCCOLLUM AND J. G. FULLER 1909 The role of inorganic phosphorus in nutrition of animals. Wisconsin Research Bull. 1.

HORIUCHI, K. 1931 Über die Phytase und die Spezifität der Phosphoesterase. J. Biochem. Tokyo, vol. 14, p. 163.

KAY, H. D. 1932 Phosphatase in growth and disease of bone. Physiol. Rev., vol. 12, p. 384.

KREIGER, C. H. 1938 Phytase and other phosphatases of the gastrointestinal tract. Thesis submitted for Master of Science degree University of Wisconsin.

KREIGER, C. H., AND H. STEENBOCK 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutrition, vol. 20, p. 125.

LOWE, J. T., AND H. STEENBOCK 1936 CLXI. Cereals and rickets. VIII. The hydrolysis of phytin in the intestines. Biochem. J., vol. 30, p. 1991.

MCCANCE, R. A., AND E. M. WIDDOWSEN 1944 Activity of the phytase in different cereals and its resistance to dry heat. Nature, vol. 153, p. 650.

MCCOLLUM, E. V., AND E. B. HART 1908 On the occurrence of a phytin-splitting enzyme in animal tissues. J. Biol. Chem., vol. 4, p. 497.

MELLANBY, E. 1944 Phytic acid and phytase in cereals. Nature, vol. 154, p. 394.

PATWARDHAN, N. V. 1937 LXXVIII. The occurrence of a phytin-splitting enzyme in the intestines of albino rats. Biochem. J., vol. 31, p. 560.

PEDERSEN, J. G. A. 1941 Experimental rickets in swine. The significance of the phytin and phytase content of the feed. Chem. Zentr., vol. 2, p. 1037.

PLIMMER, R. H. A. 1913 IV. The metabolism of organic phosphorus compounds. Their hydrolysis by the action of enzymes. Biochem. J., vol. 7, p. 43.

POTTER, V. R., AND C. A. ELVEHJEM 1936 A modified method for the study of tissue oxidations. J. Biol. Chem., vol. 116, p. 495.

RAPOPORT, S., E. LEVA AND G. M. GUEST 1941 Phytase in plasma and erythrocytes of various species of vertebrates. J. Biol. Chem., vol. 139, p. 621.

SINGSEN, E. P., AND H. H. MITCHELL 1944 Soybean meal chick rations need no inorganic phosphorus supplements. Poultry Sci., vol. 23, p. 152.

SPITZER, R. R., AND P. H. PHILLIPS 1945 The availability of soybean oil meal phosphorus to the rat. J. Nutrition, vol. 30, p. 117.

SUZUKI, U., K. YOSHINUIRA AND M. TAKAISKI 1906 Ueber ein Enzym phytase, "das, anhydro-oxy-methylendiphosphorsäure" spalet. Tokyo Imp. Univ. Coll. Agric. Bull., vol. 7, p. 503.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

III. THE EFFECT OF DIETARY ASCORBIC ACID OXIDASE [1]

MELVIN HOCHBERG, DANIEL MELNICK AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Wokes and Organ ('43) have reviewed the literature on the presence of ascorbic acid oxidase in plant tissues and the rapidity with which it destroys the ascorbic acid present. The tests conducted by Hochberg, Melnick and Oser ('43) on a homogenized composite of a daily ration of foods commonly consumed by man indicate that the activity of the ascorbic acid oxidase derived from vegetables is not materially inhibited by the presence of other foods free from this enzyme. Complete conversion of reduced to dehydroascorbic acid occurred during the period required for the homogenization of the diet, even under conditions which would be expected to inhibit oxidation of the vitamin. In the presence of molecular oxygen the oxidation of ascorbic acid may proceed beyond the dehydro stage to diketogulonic acid (Levcowich and Batchelder, '42; Penney and Zilva, '43) to oxalic acid (Dodds and Gallimore, '32; Rosenfeld, '43) and possibly to other nutritionally inactive compounds.

Young and McHenry ('42) have drawn comparisons between the calculated and determined amounts of ascorbic acid in a series of meals. The analytical values were only 24 to 75% of the theoretical median as calculated from three sets of reference tables dealing with food composition. They point out that determinations of vitamin content of fresh foods may provide little information as to the amounts in the foods as consumed, since there may be variations in duration and conditions of storage, cooking procedures, and other factors. It seemed possible, therefore, that ascorbic acid oxidase, being responsible in large measure for the lower-than-expected ascorbic acid content of foods about to be eaten, may operate in vivo, thus reducing the physiological effectiveness of the ingested vitamin.

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

In the present study the influence of ascorbic acid oxidase upon the availability of dietary and extra ascorbic acid was investigated. In vitro experiments were also conducted. The photometric method of Hochberg, Melnick and Oser ('43) was employed for the determination of both dietary and urinary ascorbic acid, measurements being made of both reduced and dehydroascorbic acid. The test subjects were the same as those previously employed (Melnick, Hochberg and Oser, '45). The basal diet, however, was purposely modified to include a greater

TABLE 1

In vitro tests of the activity of dietary ascorbic acid oxidase.

EXPERIMENT		ASCORBIC ACID FOUND IN DIET		
No.	Conditions [1]	Reduced	Dehydro	Total
		mg.	*mg.*	*mg.*
I	Blanched,[2] homogenized basal diet	101	13	114
II	I incubated 6 hrs. at 37°C.	65	27	92
III	I + 200 mg. of ascorbic acid incubated 6 hrs. at 37°C.	240	39	279
IV	Unblanched, homogenized basal diet	0	112	112
V	IV incubated 6 hrs. at 37°C.	1	44	45
VI	IV + 200 mg. of ascorbic acid incubated 6 hrs. at 37°C.	128	78	206

[1] The homogenized mixtures were incubated at their natural pH of 5.5 in open beakers and were stirred several times every half hour.

[2] The solid items in the ration were subdivided into particles, approximately 1 cubic inch, and immediately dropped into the boiling milk (previously deaerated), and the boiling continued for a 20-minute period under nitrogen. The mixture was then cooled in an ice bath and homogenized, both under nitrogen. The pH of the suspension was 5.5 throughout.

amount of raw vegetables since they contribute ascorbic acid oxidase.[2] The reason for this change was to allow a more measurable destruction of the vitamin to occur in vivo if such should be the case. The modifications in the diet were planned to permit an increase in ascorbic acid oxidase content without changing the values for the proximate constituents or vitamin content.

EXPERIMENTAL PART

In vitro tests. In table 1 are presented the results of in vitro experiments on the activity of dietary ascorbic acid oxidase. Two series of tests were conducted, both on the homogenized basal diet. In one series,

[2] The ration included bread, butter, steak, milk, eggs, sugar, orange, banana, apple, lettuce, tomato, cucumber, and a salad of raw cabbage, green beans, pepper, and carrot.

the diet [3] was first blanched in order to inactivate all enzymes present. In the other, the unblanched homogenized ration was employed. The dietary mixtures were then incubated in open beakers for a period of 6 hours at 37°C. at their natural pH of 5.5, with gentle stirring every ½ hour.

The results summarized in table 1 indicate that during the incubation of a dietary mixture after heat-inactivation of all enzymes, small but appreciable loss of ascorbic acid occurred. That this was due to oxidation may be inferred from the fact that the dehydroascorbic acid value had increased. These losses in ascorbic acid appear to be absolute since added ascorbic acid (experiment III) was almost quantitatively recovered in the reduced state from the dietary mixture.

Simple homogenization of the unheated dietary mixture resulted in marked oxidation of the ascorbic acid to dehydroascorbic acid, but with only slight oxidation beyond that stage. However, during incubation of the dietary mixture at 37°C., fully 60% of the dehydroascorbic acid was converted to non-biologically active forms. When extra ascorbic acid was added to the dietary mixture, much of the added vitamin was oxidized to and some even beyond the dehydroascorbic acid stage.

The results of the in vitro tests support the hypothesis advanced by Green ('41) that ascorbic acid oxidase catalyzes the oxidation of ascorbic acid in an absolute manner, i.e., the rate of oxidation being independent of the concentration of the vitamin. Since factors are known to be present in the gastrointestinal tract which can destroy enzymes or render them inactive, it was necessary to determine by direct studies on man what effect the ascorbic acid oxidase might have on the availability of the vitamin when cell rupture occurs in the gastrointestinal tract.

In vivo study. In table 2 are shown the results of the study on human subjects. The control data were obtained with the basal ration previously heated to inactivate all enzymes present.

During the first 3 days no extra ascorbic acid was taken; on the fourth day a test dose of 200 mg. of the vitamin was administered; and on the fifth day the basal ration alone was again ingested. The responses of the subjects were quite uniform in respect to reproducibility in their

[3] The solid constituents of the ration were subdivided into small particles (about 1 cubic inch) and dropped immediately into boiling milk previously deaerated by a stream of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized in a Waring blendor under nitrogen. In the case of the unblanched diet the homogenization was carried out under air.

basal ascorbic acid excretion, response to the test doses, and small carry-over (second 24-hour) excretions. Two weeks later the experiment was repeated, but this time the diet was consumed with the vegetables in their natural raw state. No initial or progressive decreases in the basal urinary excretion values were noted when the subjects received the unheated diet. After the postprandial test dose of 200 mg. of ascorbic acid, an increase in the urinary excretion of the vitamin resulted, followed by a small carry-over during the subsequent 24-hour period. The extra excretion of ascorbic acid following dosage was comparable to that observed during the control period when no active ascorbic acid oxidase remained in the foods taken.

TABLE 2

Urinary excretion of dietary and extra ascorbic acid by subjects receiving diets containing active and heat-inactivated[1] *ascorbic acid oxidase.*

(All values in milligrams per 24 hours.)

SUBJECT	DIET WITH ENZYMES HEAT-INACTIVATED				DIET CONTAINING ACTIVE ENZYMES				
	Basal excretions		After 200 mg. test dose of ascorbic acid		Basal excretions			After 200 mg. test dose of ascorbic acid	
	1st 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.	1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.
J.C.	56	44	141	86	36	45	57	141	69
E.M.	52	60	195	88	74	61	59	172	94
D.M.	63	55	135	75	40	44	43	163	84
M.H.	51	53	165	90	67	59	57	199	97
H.H.	12	14	66	39	22	23	27	103	42
Average	47	45	140	76	48	46	48	156	77

[1] The enzymes in the diet were inactivated by heat essentially as in the in vitro studies (see footnote to table 1) except that the blanched mixture was not homogenized.

The values shown in table 2 are for total ascorbic acid. However, in the course of the urine analyses, reduced ascorbic acid (and by difference, dehydroascorbic acid) was also determined. These data are not included in table 2 since they indicate no significant difference in the urinary excretion of either form of the vitamin during the control and test periods. In all cases approximately 91% of the total vitamin in the urine was reduced ascorbic acid, the remainder being in the dehydro state. This is in fairly good agreement with the 82% figure reported by Berryman and associates ('44) for the fraction found in the reduced form in urine after dosage with the reduced ascorbic acid.

In table 3 the data of table 2 are restated in terms of per cent of test dose excreted when taken with the diets containing active and heat-

inactivated enzymes, respectively. In considering these data it should be pointed out that somewhat greater individual variations were observed from control to test period than in the case of other ascorbic acid assays conducted with the same subjects. However, group averages are reproducible (Melnick, Hochberg and Oser, '45). The average 48-hour extra excretion of ascorbic acid when the added vitamin was consumed along with the diet in which all enzymes had been heat-inactivated was found to be 63% of the dose. A value of 71% was obtained when the vitamin was taken with the natural diet, i.e., in the presence of active ascorbic acid oxidase. The difference is not statistically significant ($t = 0.84$). It is apparent from these data that no demonstrable

TABLE 3

Stability in vivo of dietary and extra ascorbic acid in the presence of natural ascorbic acid oxidase.

SUBJECT	DIET WITH ENZYMES HEAT-INACTIVATED			DIET CONTAINING ACTIVE ENZYMES		
	Average basal excretion	Fraction of 200 mg. test dose excreted		Average basal excretion	Fraction of 200 mg. test dose excreted	
		1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.
	mg./24 hrs.	%	%	*mg./24 hrs.*	%	%
J.C.	50	46	18	46	48	12
E.M.	56	70	16	65	54	15
D.M.	59	38	8	42	61	21
M.H.	52	57	19	61	69	18
H.H.	13	27	13	24	40	18
Average	46	48	15	48	54	17
Average total excretion of extra ascorbic acid		62.4%			71.2%	
Availability of ascorbic acid in presence of oxidase	$\frac{71.2}{62.4} \times 100 = 114 \pm 16\%$					

destruction of ascorbic acid occurred by virtue of the concomitant ingestion of active ascorbic acid oxidase. In fact two of the subjects (D.M. and H.H.) present the apparent anomaly of excreting somewhat more ascorbic acid when the active enzymes were included in the diet. However, the excretion values for these particular individuals during the control period (heat-inactivated diet) were somewhat lower than usual.

The in vitro tests (table 1) demonstrated a proportionately greater destruction to have occurred in the naturally supplied ascorbic acid because the enzyme appeared to operate in an absolute rather than a relative manner. However, the reproducibility of the basal ascorbic

acid values in this study from one period to the other also demonstrates inactivity of ascorbic acid oxidase in vivo.

It is conceivable that in the gastrointestinal tract some oxidation of ascorbic acid to dehydroascorbic acid may have occurred since this change would not be detected by the assay technic. No differences occur in the form and amount of ascorbic acid excreted in the urine following dosage with reduced or dehydroascorbic acid (Johnson and Zilva, '34). The inability to determine by the bioassay technic this "incipient" oxidation of reduced ascorbic acid is unimportant nutritionally inasmuch as the organism can utilize dehydroascorbic acid equally well.

SUMMARY

In vitro tests have emphasized the rapidity with which the ascorbic acid oxidase in vegetables can oxidize ascorbic acid to and beyond the dehydroascorbic stage. Simple incubation of the dietary mixture at body temperature for a period of 6 hours resulted in a loss of approximately 60% of the biologically active vitamin. The amount of enzyme present in the homogenized diet was sufficient to catalyze oxidation of added ascorbic acid to and beyond the dehydroascorbic acid stage.

The results of the human availability study indicate that no greater destruction of either the naturally occurring ascorbic acid or of the extra ascorbic acid ingested takes place in vivo prior to absorption. Apparently ascorbic acid oxidase is destroyed or its activity is inhibited in the gastrointestinal tract. Oxidation of the vitamin to dehydroascorbic acid may have occurred but in view of the biological activity of this partially oxidized form of the vitamin, this is of no importance nutritionally.

LITERATURE CITED

BERRYMAN, G. H., C. E. FRENCH, H. A. HARPER AND H. POLLACK 1944 Response to the intravenous injection of ascorbic acid as indicated by the urinary excretion of the total and reduced forms. J. Nutrition, vol. 27, p. 309.

DODDS, E. C., AND E. J. GALLIMORE 1932 The determination of small quantities of oxalic acid. Biochem. J., vol. 26, p. 1242.

GREEN, D. E. 1941 Mechanism of Biological Oxidations. Cambridge University Press, London, England.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1943 Photometric determination of reduced and total ascorbic acid. Ind. Eng. Chem., Anal. Ed., vol. 15, p. 182.

JOHNSON, S. W., AND S. S. ZILVA 1934 The urinary excretion of ascorbic and dehydroascorbic acids in man. Biochem. J., vol. 28, p. 1393.

LEVCOWICH, T., AND E. L. BATCHELDER 1942 Ascorbic acid excretion at known levels of intake as related to capillary resistance, dietary estimates, and human requirements. J. Nutrition, vol. 23, p. 399.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

PENNEY, J. R., AND S. S. ZILVA 1943 The determination of 2, 3-diketo-l-gulonic acid. Biochem. J., vol. 37, p. 39.

ROSENFELD, B. 1943 The irreversible transformation of dehydroascorbic acid. J. Biol. Chem., vol. 150, p. 281.

WOKES, F., AND J. C. ORGAN 1943 Oxidizing enzymes and vitamin C in tomatoes. Biochem. J., vol. 37, p. 259.

YOUNG, C. M., AND E. W. MCHENRY 1942 Errors in the calculation of the nutritive value of food intake. II. Comparison of calculated and determined amounts of ascorbic acid. Can. Pub. Health J., vol. 33, p. 224.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

IV. THE INEFFICIENCY OF LIVE YEAST AS A SOURCE OF THIAMINE [1]

MELVIN HOCHBERG, DANIEL MELNICK AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Live compressed yeast has been widely advertised as a good source of the vitamin B complex. However, studies by Walker and Nelson ('33) showed that vitamin B in fresh yeast is not readily available for absorption in the gastrointestinal tract of the rat due presumably to the resistance of the live yeast cell to digestion (Montgomery and associates, '31).

In chemical methods of assay, the cells are ruptured by boiling the suspension of the yeast in dilute sulfuric acid, making possible measurement of the total thiamine content. We have confirmed this by rat assays (curative) of neutralized acid extracts of yeast.

Parsons and associates ('42 b) at the University of Wisconsin presented evidence of the non-availability to man of the water-soluble vitamins in fresh yeast. When the yeast was suspended in boiling water, and then fed, evidence of thiamine absorption was obtained. In only one type of live compressed yeast was the thiamine present in utilizable form (Parsons and collaborators, '45). This sample contained five times as much thiamine, but otherwise was identical with one of the yeast cakes previously shown to contain thiamine in a non-available form. The authors concluded that the extent to which thiamine was available in live yeast was determined not by the strain of yeast, but by the high concentration of thiamine in the yeast cells.

In the present study, the availability to man of thiamine in the live yeast was investigated by means of the human availability technic described by Melnick, Hochberg and Oser ('45). Thiamine intake was determined by the thiochrome procedure (Hennessy, '41), and urinary excretion by the colorimetric method (Hochberg and Melnick, '44).

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

Thiamine was the only vitamin included in this study, since according to the work of Parsons and associates ('42) its excretion is an index of the degree of availability of all the water-soluble vitamins present. Parallel in vitro studies were also conducted.

In vitro tests

It has long been known that, during growth and multiplication, yeast cells are able to remove thiamine from solution. Thus, brewers' yeast is rich in thiamine while the beer contains only a small fraction of the vitamin originally present in the wort. Thiamine is present in yeast primarily in the phosphorylated form. During the drying of yeast cells a phosphatase is liberated which converts some of the combined thiamine to the free form (Melnick and Field, '39). Therefore aid-dried

TABLE 1

Free and phosphorylated thiamine in commercial samples of moist compressed yeast. (All values in micrograms per gram.)

SAMPLE		THIAMINE FOUND			
No	Description	Non-phosphorylated	Phosphorylated	Total	Per cent Phosphorylated
1	Regular yeast	0.3	3.9	4.2	93
2	Regular yeast	0.5	4.6	5.1	90
3	High vitamin yeast	50	164	214	77
4	High vitamin yeast	47	152	199	76
5	High vitamin yeast	64	13	77	17

yeast samples may contain variable proportions of thiamine in the phosphorylated state, depending on the conditions of preparation. In the present report the differences in the thiamine values before and after treatment of the test extracts with phosphatase are attributed to the presence of phosphorylated thiamine.

In table 1 are presented the results obtained in the analyses of five samples of compressed yeast cakes. Two of these were bakers' yeast, available for commercial use but not enriched with added vitamins. Preparations 3 and 4 were "high vitamin" yeast preparations employed at the time in the manufacture of enriched bread. The fifth sample was a high vitamin yeast cake sold directly to the public as a source of the water-soluble vitamins. The data indicate that, in the non-enriched yeast samples, approximately 90% of the thiamine was present in the phosphorylated state. In the high vitamin yeast used for baking purposes, about three-fourths of the vitamin was found in the

combined form. In the sample intended for direct human consumption, only about one-sixth of the vitamin was phosphorylated.

In table 2 are presented the results of experiments to determine the fate of thiamine added to an aqueous suspension of live yeast cells. For these experiments, the non-enriched variety of yeast was employed. Six yeast cakes weighing 75 gm. were dispersed in 360 ml. of acetate buffer at pH 4.5 to make a uniform suspension. This was then stored at 37°C. for 6 hours with rotatory agitation every ½ hour. At the end of the 6-hour period the suspensions were centrifuged and assays conducted on the washed yeast sediment and on the pooled supernatant

TABLE 2

Absorption and phosphorylation of thiamine by yeast cells[1] *in aqueous suspension:*

SAMPLE	THIAMINE FOUND, PER ENTIRE SAMPLE			
	In solution	In yeast sediment		
	Non-phosphorylated	Non-phosphorylated	Phosphorylated	Total
75 gm. yeast[2]	12 µg. 4%	10 µg. 3%	290 µg. 93%	300 µg. 96%
75 gm. yeast + 1000 µg. thiamine	7 µg. 0.5%	36 µg. 3%	1152 µg. 97%	1168 µg. 100%

[1] Sample no. 1 of table 1.

[2] The weight of 6 commercial yeast cakes is 75 gm. Uniform suspensions in 360 ml. of acetate buffer at pH 4.5 were prepared and stored at 37°C. for 6 hours. These were stirred every ½ hour. The suspensions were centrifuged and assays conducted on the washed yeast sediment and on the pooled supernatant phase plus washings.

phase plus the washings. These tests were repeated on another sample of the suspended yeast cells to which had been added 1000 µg. of synthetic free thiamine.

Practically all the vitamin originally in solution was found to be removed by the yeast cells. Furthermore, almost all of the thiamine in the yeast sediment was in the phosphorlylated state. This removal of thiamine from solution and its phosphorlylation by the live yeast cell is confirmatory of the results obtained in these laboratories on the fate of thiamine added for enrichment of bread (Melnick and associates, '41). It was found that during the fermentation of the dough, yeast phosphorylated the synthetic free thiamine added for enrichment, so that the baked bread contained the same percentage of the total thiamine in the phosphorylated form[2] as when natural combined thiamine was used for the fortification.

[2] Confirmation that this was indeed phosphorylated thiamine was obtained by testing specifically for cocarboxylase by the Lohmann and Schuster ('37) mannometric procedure.

The present experiments on the phosphorylating ability of yeast are of particular value since they definitely indicate that the thiamine removed from solution is not merely adsorbed on the cell wall but actually passes into the cell proper. This would seem to explain in part the mechanism responsible for the low availability of thiamine to human subjects as reported by Parsons and associates ('42a, '42b). Because better availability was observed by the Wisconsin workers (Parsons and associates, '45) from live yeast containing high proportions of thiamine, the in vitro tests were extended to studies of the enriched yeast sample recommended for direct consumption. From the values given in table 1, it was apparent that this yeast differed from the others in that most of its thiamine was present in the free state.[3] Analyses were then conducted on the suspension of the live yeast cells before and after the period of incubation at 37°C. Furthermore, the vitamin in solution was partitioned into free and phosphorylated thiamine.

The results shown in table 3 demonstrate that in the supernatant solution practically all the vitamin was present in the free state. During the period of storage at 37°C., the proportion of thiamine retained by the yeast sediment increased from 28 to 52%. This was due to a rise in concentration of both the free and phosphorylated vitamin. When extra thiamine was added to the suspension of live yeast cells no increase was observed in the absolute quantity of thiamine in the yeast sediment, either as the free or phosphorylated vitamin.

The presence of the thiamine in this yeast sample primarily as the free vitamin, the inability of these live yeast cells to phosphorylate thiamine to any significant extent and the large overage in thiamine content (i.e., above that indicated on the label) suggested that at least the claimed quantity of thiamine in the product might be available to the human. It was suspected that this might represent an attempt on the part of the manufacturer to solve the problem of the unavailability of thiamine in the live yeast cell. These factors might explain the favorable results obtained by Parsons and associates ('45) when live yeast cells containing large quantities of thiamine were fed.

In vivo tests

Human availability studies were conducted on the yeast sample used in the experiment summarized in table 3. Six yeast cakes (each 12.5 gm.) were taken, suspended in milk, two after each meal. During the control

[3] The actual thiamine content of the sample as determined by chemical analysis was almost twice as great as claimed for the product, namely, 1.92 mg. instead of 1.0 mg. per two yeast cakes.

TABLE 3

In vitro absorption and phosphorylation of thiamine by enriched live yeast[1] *in aqueous suspension.*

EXPERIMENT	THIAMINE FOUND, PER ENTIRE SAMPLE					
	In solution			In yeast sediment		
	Non-phosphorylated	Phosphorylated	Total	Non-phosphorylated	Phosphorylated	Total
75 gm. yeast[2]	....	....	....	4.80 mg. 83%	0.96 mg. 17%	5.76 mg. 100%
75 gm. yeast suspended in acetate buffer at pH 4.5 at 25°C. for 2 min.	3.90 mg. 71%	0.09 mg. 2%	3.99 mg. 72%	1.08 mg. 20%	0.45 mg. 8%	1.53 mg. 28%
75 gm. yeast suspended in acetate buffer at pH 4.5 and stored at 37°C. for 6 hrs.	2.55 mg. 42%	0.36 mg. 6%	2.91 mg. 48%	2.34 mg. 38%	0.84 mg. 14%	3.18 mg. 52%
75 gm. yeast + 5.76 mg. thiamine suspended in acetate buffer at pH 4.5 and stored at 37°C. for 6 hrs.	7.59 mg. 68%	0.30 mg. 3%	7.89 mg. 71%	2.46 mg. 22%	0.78 mg. 7%	3.24 mg. 29%

[1] Sample no. 5 of table 1. [2] Six yeast cakes.

TABLE 4

Rate and degree of urinary excretion of thiamine following ingestion of the vitamin in solution and in yeast.

(All values in milligrams per day.)

SUBJECT	THIAMINE IN SOLUTION			THIAMINE IN YEAST CAKES		
	Basal	After 5.76 mg. of thiamine in solution[1]		Basal	After 6 live yeast cakes[2]	
		1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.
J.C.	0.26	1.78	0.57	0.23	0.70	0.40
E.M.	0.20	1.67	0.64	0.28	0.37	0.29
D.M.	0.20	1.56	0.55	0.25	0.44	0.25
M.H.	0.20	1.68	0.59	0.24	0.56	0.37
H.H.	0.17	1.48	0.45	0.21	0.30	0.22
Average	0.21	1.63	0.56	0.24	0.47	0.31

[1] Taken as three 1.92 mg. doses of thiamine in milk, one dose at the end of each meal.

[2] Containing 5.76 mg. of thiamine. Two cakes, containing 1.92 mg., were suspended in milk and taken at the end of each meal.

period, the same quantity of pure thiamine was taken dissolved in milk, one-third of the dose being given after each meal. Each dose was 1.92 mg. of thiamine, the total being 5.76 mg. per subject per day. The results obtained in this study are summarized in table 4.

The basal urinary excretion values for the five test subjects were of the same magnitude during the control and test periods. However, the marked increases in the urinary excretion values following the taking of thiamine in solution were not observed when the test dose of thiamine was taken as the live yeast supplement. Inasmuch as one dose was taken after the evening meal, it was considered advisable to collect the urine samples for an additional 24-hour period in order to be sure that an erroneous conclusion regarding availability would not

TABLE 5

Availability of thiamine in yeast.

SUBJECT	PORTION OF TEST DOSE EXCRETED					
	Control period, thiamine in solution			Test period, thiamine in yeast		
	1st 24 hours	2nd 24 hours	Total 48 hours	1st 24 hours	2nd 24 hours	Total 48 hours
	%	%	%	%	%	%
J.C.	26.4	5.4	31.8	8.2	3.0	11.2
E.M.	25.5	7.6	33.1	1.6	0.2	1.8
D.M.	23.6	6.1	29.7	3.3	0	3.3
M.H.	25.7	6.8	32.5	5.6	2.3	7.9
H.H.	22.7	4.9	27.6	1.6	0.2	1.8
Average	24.8	6.2	30.9	4.1	1.1	5.2

Availability of thiamine in yeast $= \frac{5.2}{30.9} \times 100 = 17\%$.

be due simply to a slower rate of absorption of the thiamine derived from the intact yeast cells. Examination of the data indicates that the "carry-over" effect was not responsible for the lower thiamine excretion during the first 24 hours.

In table 5, the results in the preceding table are restated on a percentage excretion basis. The urinary values demonstrate that approximately 25% of the pure solution dose was excreted during the 24 hours following the taking of the first supplement, and 6% more during the second 24-hour period for a total excretion of 31%. After ingestion of the live yeast cakes, the quantities of extra thiamine found in the urine were exceedingly small; 4% of the dose during the first 24 hours and 1% during the second 24 hours. Thus, the physiologically available thiamine in live yeast is calculated to be only 17%, or 33% of that

claimed for this particular product, despite the fact that the greater portion of its thiamine was in the free state and that the material exhibited a markedly limited capacity to phosphorylate the vitamin.

The low availability cannot be attributed to a slower rate of absorption of the thiamine from live yeast with concomitant improvement in its utilization. If this were the case, one would expect the urinary response following administration of a pure solution of thiamine in three separate test doses over a period of a day to be less than that noted when the same quantity of the vitamin was taken as a single test dose. Such was not the case.[4] Furthermore, the ratio of the urinary excretion responses after taking the thiamine in the form of live yeast as compared to taking thiamine in solution during the first 24 hours, namely, 4 to 25, was exactly the same as the ratio of the carry-over values, 1 to 6%.

SUMMARY

Bakers' yeast is capable of removing large quantities of thiamine from solution. That this removal is not due to simple adsorption of the thiamine on the yeast cell wall but to actual passage of the thiamine into the cell proper, is evidenced by phosphorlylation of the vitamin. Other preparations of live yeast on the market have limited ability to phosphorylate thiamine. In these samples the major portion of the thiamine is found in the free state.

Despite the facts that (a) most of the thiamine in the live yeast employed in the present human availability study was present in the free form, (b) the yeast was practically ineffective in phosphorylating the vitamin and (c) there was almost a 100% overage of thiamine in the sample — all of which should favor the presence of available thiamine at least to the extent of the content of thiamine claimed for the product — only 17% of the total thiamine present was physiologically available, or 33% of the claimed potency.

The low availability value for the thiamine could not be attributed to a slower rate of absorption of the vitamin from live yeast.

ADDENDUM

Correspondence with Dr. Helen T. Parsons, University of Wisconsin, Madison, confirmed the suspicion that the yeast sample used in our availability studies was made by the same manufacturer as her sample A_2. Calculations, similar to those used in the present studies indicate that 27% of the thiamine in her yeast sample A_2 is available (see table 3, Parsons and associates, '45). Considering that different test subjects were employed subsisting on basal rations which were not

[4] See previous report (Melnick, Hochberg and Oser, '45) presenting the excretion data when thiamine was taken postprandially as a single 5.0 mg. test dose.

the same, and that different batches of the yeast were used in the assays, the values obtained by the two laboratories are considered to be in very good agreement.

LITERATURE CITED

HENNESSY, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal Ed., vol. 13, p. 216.

HOCHBERG, M., AND D. MELNICK 1944 A simple modification of the colorimetric method for routine thiamine clearance tests. J. Biol. Chem., vol. 156, p. 53.

LOHMANN, K., AND P. SCHUSTER 1937 Untersuchungen über die Cocarboxylase. Biochem. Ztschr., vol. 294, p. 188.

MELNICK, D., AND H. FIELD, JR. 1939 Chemical determination of vitamin B_1. Hydrolysis of cocarboxylase. J. Biol. Chem., vol. 127, p. 531.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

MELNICK, D., A. MABARDIE, A. BERNSTEIN AND B. L. OSER 1941 The fate of vitamin B_1 in production of baked goods. Northwestern Miller, vol. 208, p. 17.

MONTGOMERY, B. E., A. K. BOAR, L. ARNOLD AND O. BERGEIM 1931 Destruction of yeast in the normal human stomach. Proc. Soc. Exp. Biol. Med., vol. 28, p. 385.

PARSONS, H. T., AND J. COLLORD 1942 a Human utilization of thiamin and riboflavin in yeast. J. Am. Diet. Assn., vol. 18, p. 805.

PARSONS, H. T., J. COLLORD, J. GARDNER, F. M. STRONG AND W. H. PETERSON 1942 b Utilization by man of the vitamin B complex in fresh yeast. Federation Proc., vol. 1, p. 129.

PARSONS, H. T., A. WILLIAMSON AND M. L. JOHNSON 1945 The availability of vitamins from yeasts. I. The absorption of thiamine by human subjects from various types of bakers' yeast. J. Nutrition, vol. 29, p. 373.

WALKER, R., AND E. M. NELSON 1933 Fresh and dried yeast as sources of vitamin B. Am. J. Physiol., vol. 103, p. 25.

ESTIMATION OF THE RELATIVE NUTRITIVE VALUE OF VEGETABLE PROTEINS BY TWO CHEMICAL METHODS [1]

ROBERT JOHN EVANS AND J. L. ST. JOHN
Division of Chemistry, Washington Agricultural Experiment Station, Pullman, Washington

(Received for publication May 25, 1945)

Recent work of Draper and Evans ('44) has shown that different soybean oil meals may differ in protein quality as measured by the gross value method.

Almquist, Stokstad, and Halbrook ('35) developed a chemical method for estimating the quality of animal protein concentrates. Almquist ('41) stated that he did not recommend this method for vegetable protein concentrates since he had not investigated vegetable products. Olsen and Palmer ('40) compared the chemical protein quality index of Almquist with a biological value determined by the nitrogen retention method with rats. Their results with a number of animal and plant foods seem to indicate a relationship between the two methods for natural foods.

The simple vegetable proteins have been classified by Osborne ('24) according to their solubilities into the albumins (soluble in water), the globulins (insoluble in water but soluble in saline solutions), the glutelins (insoluble in water, saline solutions, or alcohol, but soluble in very dilute acids and alkalies), the prolamines (soluble in relatively concentrated alcohol), the albuminoids, the histones, and the protamines. To the knowledge of the authors, no attempt has been made to relate the relative amounts of these different proteins in a concentrate to the protein nutritive value of the concentrate.

It appeared desirable to study the relation of the nutritive values of some vegetable protein concentrates to the chemical protein quality index, and to the per cent of total nitrogen present in each of the different protein fractions defined above. It also appeared desirable to determine the influence of heat treatment upon the solubility of the soybean proteins. A determination of any chemical differences existing

[1] Published as Scientific Paper no. 653, College of Agriculture and Agricultural Experiment Station, State College of Washington, Pullman.

between the vegetable protein concentrates having high protein nutritive values and those having low protein nutritive values would be of importance from both theoretical and practical standpoints. A rapid method of estimating the relative quality of vegetable protein concentrates would also be of value.

EXPERIMENTAL

The gross protein values of ten soybean oil meals, two cottonseed meals, and two pea meals were determined with chicks by Draper and Evans ('44). The soybean oil meals were commercial products, obtained from three different sources and were prepared by expeller process, solvent process, or solvent process-uncooked. The method of preparation of two of the soybean oil meals was unknown, but from physical characteristics of the meals, they appear to have been prepared by the solvent process. The cottonseed meals were commercial products of unknown history. The pea meals were prepared by grinding cull Alaska, and First and Best peas.

Relation of chemical protein quality index to nutritive value

The chemical protein quality index was determined on these same vegetable protein concentrates by the procedure of Almquist, Stokstad, and Halbrook ('35). This method consists of a chemical determination of four protein fractions. The chemical protein quality index is calculated from these results by the following formula:

Protein quality index $= A - (B + 0.6\,C) + 0.4\,D$: Where $A =$ per cent protein precipitated by copper salts (inclusive of B and C); $B =$ per cent protein not digested with pepsin; $C =$ per cent protein soluble in hot water; and $D =$ per cent protein precipitated by phosphotungstic acid.

The values for the chemical protein quality index of the soybean oil meals ranged from 84.8 to 89.1 (table 1). Only one of these values was below 88.0. The values for the gain in weight per gram of supplementary protein consumed ranged from 3.5 to 6.6 gm. It is apparent that the growth method gave much larger differences than the chemical method. A coefficient of correlation of $+0.367$ between the chemical index of protein quality and the gain per gram of supplementary protein consumed was obtained for all of the soybean oil meals. A coefficient of 0.632 was required for significance.

The two pea meals gave as good gains in weight per gram of supplementary protein as the expeller process soybean oil meals. The chemical protein quality indexes were definitely lower for the pea meals than

for the expeller process soybean oil meals. This was the result of the high hot-water-soluble protein fraction in the pea meals.

The poorest gains in weight per unit of supplementary protein were obtained with the cottonseed meals. The protein quality indexes were also the lowest. The low protein quality index of the cotttonseed meal was the result of a large indigestible protein fraction.

A coefficient of correlation between gain per gram of supplementary protein and the chemical protein quality index of + 0.815 was obtained for all of the vegetable protein concentrates of table 1. A value of 0.661 was required to be highly significant. Leaving the three expeller process soybean oil meals out of the calculations, a coefficient of correlation of + 0.946 was obtained while 0.735 was required to be highly significant.

No differences in chemical protein quality index between expeller and solvent process soybean oil meals were observed. The solvent process meals, however, gave a greater gain in weight per gram of supplementary protein than did the expeller process meals (table 1).

Relation of protein solubility to nutritive value

The various proteins, from samples of the same vegetable protein concentrates, were peptized and the per cent of total nitrogen in each fraction determined by the method of Lund and Sandstrom ('43). The proteins of each concentrate are divided by this method into the protein soluble in water (albumins and part of globulin), the protein soluble in 5% potassium chloride solution (remainder of globulins), the protein soluble in 70% ethyl alcohol solution (prolamines), the protein soluble in 0.2% potassium hydroxide solution (glutelins), and the residual protein fractions. They will be referred to as albumin, globulin, prolamine, glutelin, and residual proteins respectively in the remainder of this paper. Part of the globulins are included in the albumin fraction because of the presence of salts in the soybean oil meal.

The per cent of total protein present as albumins, globulins, prolamines, and glutelins for each sample is presented in table 2. The per cent of residual protein and the gain per unit of supplementary protein are also included in table 2. Over half of the protein of the uncooked concentrates was in the albumin fraction. The peas contained considerable protein in the globulin fraction.

There was little difference between any of the cooked meals in their contents of albumins, globulins, or prolamines. Less than 10% of the total nitrogen was in the albumin fraction; less than 5% was in the globulin fraction; and less than 4% was in the prolamine fraction.

TABLE 1

Comparison of the chemical protein quality index and the gain per gram of supplementary protein for some vegetable protein concentrates.

SAMPLE NO.	PROTEIN CONCENTRATE	PROCESS	GAIN PER UNIT PROTEIN[1]	CRUDE PROTEIN	PER CENT OF TOTAL PROTEIN				PROTEIN QUALITY INDEX
					Copper ppt. (A)	Phosph. tung. (D)	Undi-gest (B)	Hot water soluble (C)	
			gm.	%					A — (B+.6C) — .4D
565	Soybean oil meal	Unknown	6.6	46.3	98.7	0.6	6.0	5.4	88.1
758	Soybean oil meal	Unknown	6.4	46.8	97.6	0.9	5.1	5.1	89.7
635	Soybean oil meal	Solvent	6.4	48.6	97.4	0.8	6.6	5.4	88.6
763	Soybean oil meal	Solvent	6.2	46.8	96.9	0.9	6.4	4.9	88.4
667	Soybean oil meal	Solvent	5.7	46.8	97.2	0.6	5.8	4.3	89.1
706	Soybean oil meal	Solvent	5.7	47.1	96.6	0.8	5.5	4.9	88.5
759	Soybean oil meal	Solvent — uncooked	4.9	50.0	95.7	0.8	6.6	7.8	84.8
764	Soybean oil meal	Expeller	4.9	46.1	96.5	0.9	5.0	5.9	88.5
704	Soybean oil meal	Expeller	4.6	45.3	96.0	0.7	5.3	4.4	88.3
765	Soybean oil meal	Expeller	3.5	46.6	96.4	0.9	4.7	6.0	88.4
761	Cottonseed meal	Unknown	2.8	37.1	94.2	0.8	17.2	6.5	73.3
702	Cottonseed meal	Unknown	1.2	45.0	93.3	0.7	18.9	6.7	70.7
755	Alaska peas	Uncooked	4.7	23.4	90.6	1.7	3.8	12.8	79.9
754	First and best peas	Uncooked	4.5	27.7	92.2	1.1	3.6	18.4	77.6

[1] From Draper and Evans ('44).

TABLE 2

Protein distribution in some vegetable protein concentrates.

SAMPLE NO.	PROTEIN CONCENTRATE	PROCESS	GAIN PER GRAM OF PROTEIN [2]	ALBUMINS [1]	GLOBULINS [1]	PROLAMINES [1]	GLUTELINS [1]	RESIDUAL PROTEIN [1]
			gm.	%	%	%	%	%
565	Soybean oil meal	Unknown	6.6	8.9	3.6	3.4	45.3	38.7
758	Soybean oil meal	Unknown	6.4	9.9	4.7	3.7	50.0	30.9
635	Soybean oil meal	Solvent	6.4	7.6	2.9	2.0	47.5	40.2
763	Soybean oil meal	Solvent	6.2	6.7	1.7	1.9	43.4	46.1
667	Soybean oil meal	Solvent	5.7	8.7	2.7	2.3	49.1	38.1
706	Soybean oil meal	Solvent	5.7	7.9	4.2	4.2	45.2	37.7
759	Soybean oil meal	Solvent — uncooked	4.9	75.6	5.7	3.5	6.0	9.2
764	Soybean oil meal	Expeller	4.9	9.1	1.7	2.1	34.3	52.8
704	Soybean oil meal	Expeller	4.6	6.5	2.0	2.4	26.2	62.5
765	Soybean oil meal	Expeller	3.5	9.1	1.3	2.3	31.6	54.0
761	Cottonseed meal	Unknown	2.8	8.0	4.0	1.9	8.3	77.2
702	Cottonseed meal	Unknown	1.2	8.4	19.3	2.4	9.1	60.2
755	Alaska peas	Uncooked	4.7	56.1	17.8	3.9	4.3	17.4
754	First and best peas	Uncooked	4.5	53.7	20.6	3.7	5.5	15.4

[1] Per cent of total protein.
[2] From Draper and Evans ('44).

Eight and three-tenths per cent of the total nitrogen of one sample of cottonseed meal was in the glutelin fraction. Seventy-seven and two-tenths per cent of the nitrogen of this same meal occurred in the residual protein fraction. Fifty per cent of the nitrogen of one soybean oil meal sample was in the glutelin fraction, and only 30.9% occurred in the residual protein fraction. There was a highly significant correlation for the cooked meals of + 0.928 between the grams gain in weight of chicks per gram of supplementary protein consumed and the per cent of the total protein in the glutelin fraction. There was a coefficient of correlation of — 0.788 between the gain per gram of supplementary protein and the per cent residual protein in the cooked meals. A coefficient of correlation of 0.735 was required to be highly significant.

The solvent process meals contained a higher percentage of dilute alkali soluble protein and a lower percentage of residual protein than the expeller process meals, (table 2).

The influence of heat treatment on the protein distribution in solvent process soybean flakes

Portions of soybean oil meal sample no. 759, which was an uncooked solvent process meal, were given different heat treatments. Portions

TABLE 3

The influence of heat treatment on the protein distribution in solvent process soybean flakes.

HEAT TREATMENT	TIME HEATED	ALBUMINS [1]	GLOBULINS [1]	PRO-LAMINES [1]	GLUTELINS [1]	RESIDUAL PROTEIN [1]
	min.	%	%	%	%	%
None	0	75.6	5.7	3.5	6.0	9.2
Autoclaved at 121°C.	5	49.6	6.3	2.7	19.8	21.6
Autoclaved at 121°C.	15	8.8	6.4	3.4	45.8	35.9
Autoclaved at 121°C.	30	6.4	1.6	3.2	38.6	49.6
Autoclaved at 121°C.	60	7.5	1.2	2.6	22.6	65.4
Autoclaved at 121°C.	120	8.6	1.0	2.7	10.0	77.3
Heated dry at 121°C.	60	64.4	6.8	2.3	10.1	16.5
Cooked in boiling water	60	15.2	3.6	3.6	38.2	38.6
Autoclaved at 110°C.	60	6.1	2.2	3.0	44.2	43.6

[1] Per cent of total protein.

were autoclaved at 121°C. for 5, 15, 30, 60, and 120 minutes. One portion was heated dry at 121°C., another was cooked wet by heating in a beaker in a boiling water bath, and another was autoclaved at 110°C. for 60 minutes. The proteins were divided into five fractions as described under the preceding section.

The results are presented in table 3. Heating decreased the percentage of albumins and globulins present in the soybean oil meal. The

glutelins were first increased and then decreased by heat treatment. The residual or insoluble protein was increased by heat treatment. It is apparent from the changes in solubility caused by heating, that the glutelin and residual protein fractions were composed largely of denatured proteins. A slight heat denaturation produced a protein insoluble in water or 5% potassium chloride, but soluble in 0.2% potassium hydroxide. Further heat denaturation produced a protein that was insoluble in 0.2% potassium hydroxide.

Dry heat was less effective than wet heat in causing a heat denaturation of the soybean proteins. The amount of heat denaturation was increased both by increasing the temperature of heating and increasing the time of heating.

DISCUSSION

The chemical protein quality index was found satisfactory as a chemical method of evaluating the protein nutritive values of animal protein concentrates (Almquist, Stokstad, and Halbrook, '35; Almquist, '41; Evans, Carver, and Draper, '44). The data presented here indicate that it may be of use in evaluating vegetable protein concentrates if the products are not overcooked. The three expeller process soybean oil meals and the two cottonseed meals appear to have been definitely overlooked as shown by the high content of residual protein. The same relation between protein quality index and supplementary protein nutritive value does not apply to the vegetable protein concentrates as it does to the animal concentrates. The lowest chemical protein quality index obtained in this experiment was 70.7 for cottonseed meal. The Alaska peas had a chemical protein quality index of about 80 and gave a gain in weight of 4.7 gm. per gram of supplementary protein consumed. Evans, Carver, and Draper ('44) obtained similar values for the chemical protein quality index of commercial herring fish meal, but the gain in weight per gram of supplementary protein consumed was 8.3 gm. One dogfish meal having a chemical protein quality index of only 44.5 gave a gain in weight of 5.7 gm. per gram of supplementary protein consumed. It is therefore evident that animal protein concentrates cannot be compared with vegetable protein concentrates by the chemical protein quality index proposed by Almquist, Stokstad, and Halbrook ('35).

The chemical protein quality index was not a satisfactory means of detecting differences in supplementary nutritive value between different soybean oil meals. It was first believed that the differences in supplementary nutritive value of different soybean oil meals were due to dif-

ferences in amino acid content of the meals. Since methionine has been shown to be the amino acid most likely to be lacking in soybean oil meals (Almquist, Mecchi, Kratzer, and Grau, '42), the sulfur distribution in these meals was determined. No differences were observed, however, in the cystine or methionine contents of the meals (Evans, '45 b) or in the per cent of organic sulfur dissolved by digestion with pepsin (Evans, '45 a).

It was found in the present study that there was a considerable difference between the different soybean oil meals with regard to the per cent of protein peptized by the different solvents. The cooked meals contained only one-tenth as much albumin as the uncooked meals. The heat-treated soybean oil meals having the highest percentage of glutelin gave the greatest gains in weight per unit of supplementary protein. Those having the least glutelin gave the least gains.

The differences in supplementary nutritive values of the different soybean oil meals appear to have been due to differences in degree of denaturation of the soybean proteins by heat treatment. The per cent of protein in the glutelin fraction was increased by short periods (up to 15 minutes) of autoclaving at 121°C., but was decreased by more prolonged heating. The curve obtained was very similar to the one presented by Bird and Burkhardt ('43) relating time of autoclaving to chick weight at 9 weeks of age. The effect of heat on the per cent of glutelin and the protein nutritive value is being further investigated. How heating and denaturation of the protein influences protein availability for the growing chick has yet to be determined, but is being investigated.

SUMMARY

A comparison was made between gain in weight per unit of supplementary protein as determined by Draper and Evans ('44) on ten samples of commercial soybean oil meal, two of cottonseed meal, and two of cull peas, and the chemical protein quality index (Almquist, Stokstad, and Halbrook, '35). A comparison between the gain in weight of chicks per unit of protein and the per cent protein present as albumin, globulin, prolamine, glutelin, and residual protein was also made.

The chemical protein quality index appeared to give good indication of the relative protein nutritive value of the vegetable protein concentrates studied with the exception of the overcooked soybean oil meals. A coefficient of correlation between the gain per unit of supplementary protein and the chemical protein quality index of + 0.946 was obtained

for all of the concentrates studied except the three expeller process soybean oil meals.

A high coefficient of correlation of + 0.928 between the gain per unit of supplementary protein and the per cent glutelin was obtained for the heat treated concentrates. A determination of the per cent glutelin may offer a chemical means of estimating differences in the protein nutritive value of soybean oil meals and, in combination with the per cent residual protein, the extent of heat denaturation of the soybean proteins.

LITERATURE CITED

ALMQUIST, H. J. 1941 Chemical estimation of quality in animal protein concentrates. J. Nutrition, vol. 21, p. 347.

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, vol. 24, p. 385.

ALMQUIST, H. J., E. L. R. STOKSTAD AND E. R. HALBROOK 1935 Supplementary values of animal protein concentrates in chick rations. J. Nutrition, vol. 10, p. 193.

BIRD, H. R., AND G. J. BURKHARDT 1943 Factors affecting the nutritive value of soybean oil meals and soybeans for chickens. Maryland Agr. Expt. Sta., Bull. A27, p. 35.

DRAPER, C. I., AND R. J. EVANS 1944 Plant protein concentrates in the chick ration. Poultry Sci., vol. 23, p. 507.

EVANS, R. J. 1945 a The sulfur content of the pepsin digestible fraction of protein concentrats. Arch. Biochem., vol. 7, p. 33.

——— 1945 b Unpublished results.

EVANS, R. J., J. S. CARVER AND C. I. DRAPER 1944 A comparison of the chemical protein quality index with the gross protein value of fish protein concentrates. Arch. Biochem., vol. 3, p. 337.

LUND, A. P., AND W. M. SANDSTROM 1943 The proteins of various tree seeds. J. Agr. Research, vol. 66, p. 349.

OLSON, F. C., AND L. S. PALMER 1940 Comparison of a chemical and a biochemical method for determining the biological value of proteins and an evaluation of the endogenous nitrogen. J. Agr. Research, vol. 60, p. 331.

OSBORNE, T. B. 1924 The Vegetable Proteins. Longmans, Green and Co., London.

OBSERVATIONS ON MONOMETHYLAMINOETHANOL AND DIMETHYLAMINOETHANOL IN THE DIET OF CHICKS

T. H. JUKES, J. J. OLESON AND A. C. DORNBUSH
Lederle Laboratories, Pearl River, New York

(Received for publication June 2, 1945)

It was noted recently (Jukes and Oleson, '45) that dimethylaminoethanol had a choline-like nutritional effect in promoting growth and preventing perosis in chicks on a diet deficient in choline. The present communication reports experiments with mono- and di- methylaminoethanol as related to growth and the prevention of perosis in chicks.

EXPERIMENTAL

Day-old New Hampshire Red chicks were placed in electrically-heated battery brooders and were fed the experimental diets immediately. Ten chicks were used in each group. The chicks were weighed weekly and were examined for perosis at frequent intervals. Basal diet 1 had the following composition: Glucose,[1] 56 parts; alcohol-extracted casein, 18; dried yeast,[2] 5; corn oil,[3] 5; gelatin, 5; gum arabic, 5; salt mixture ($CaCO_3$, 23 parts; K_2HPO_4, 10; KH_2PO_4, 10; NaCl, 17; bone ash, 30; $MgSO_4$, 5; ferric citrate, 3.5; $MnSO_4 \cdot 4H_2O$, 1; copper carbonate, 0.1; zinc carbonate, 0.1; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.5; KI, 0.8; cobalt acetate, 0.005; nickel carbonate, 0.002) 5. The birds were dosed three times weekly with 0.1 ml. of a solution of fat-soluble vitamins in corn oil. Each 0.1 ml. contained vitamin A, 7000 units; vitamin D_3,[4] 400 units; mixed tocopherols (34% solution), 20 mg.; 2-methyl-1, 4 naphthoquinone, 5 µg.

In the first experiment various supplements were added to the diet as summarized in table 1. The weights of choline refer to the chloride, of betaine to the hydrochloride and of mono- and di- methylaminoethanol to the free bases,[5] which were neutralized with hydrochloric acid before adding them to the diets.

[1] Cerelose.

[2] Anheuser-Busch, Strain G.

[3] Mazola.

[4] Delsterol.

[5] A sample of monomethylaminoethanol was kindly synthesized by Dr. J. Semb, and a second sample was furnished by the American Carbon and Carbide Company.

The observations in table 1 indicate that both monomethylaminoethanol and dimethylaminoethanol were effective in preventing perosis. Growth was stimulated by dimethylaminoethanol but not by monomethylaminoethanol. Methionine at the level fed was completely ineffective in preventing perosis or promoting growth as previously noted (Jukes, '41) but methionine stimulated growth when fed in combination with either monomethylaminoethanol or dimethylaminoethanol. There was an indication that methionine might stimulate growth slightly when added to the basal diet plus 0.2% choline. The possibility of a slight deficiency of methionine in diet 1 is not excluded upon comparing its probable methionine and cystine content with the

TABLE 1

Effect on growth and perosis in chicks produced by adding various compounds to basal choline-deficient diet 1.

ADDITION TO 100 GM. BASAL DIET 1	INCIDENCE OF PEROSIS AT 21 days	INCIDENCE OF PEROSIS AT 28 days	GAIN IN 28 DAYS
	%	%	gm.
None	60	80	113
0.2 gm. methionine	90	90	112
0.2 gm. monomethylaminoethanol	0	0	89
0.2 gm. methionine plus 0.2 gm. monomethylaminoethanol	0	0	123
0.2 gm. dimethylaminoethanol	0	0	147
0.2 gm. methionine plus 0.2 gm. dimethylaminoethanol	0	0	181
0.2 gm. choline	10	10	184
0.2 gm. methionine plus 0.2 gm. choline	0	0	196

requirement of the chick for these amino acids (Grau and Almquist, '43). The "choline" content of diet 1 was found to be approximately 0.018% by the Neurospora assay method (Horowitz and Beadle, '43).

A diet deficient in choline and methionine (diet 2) was prepared by modifying diet 1 as follows: 23 parts of "Alpha protein"[6] was substituted for the gelatin and casein, the yeast was reduced to 3 parts, the glucose was increased to 59 parts and the following synthetic vitamins were added per 100 gm. of diet: Thiamine, 1 mg.; riboflavin, 1 mg.; pyridoxine, 1 mg.; niacinamide, 5 mg.; calcium pantothenate, 10 mg.; inositol, 50 mg.; and biotin, 10 μg. "Alpha protein," derived from soybeans, has been described by Grau and Almquist ('43) as ". . . con-

[6] A commercial soya protein furnished by the Glidden Company.

taining 86.6% protein (N × 6.25) and 1.75% ash. The protein contained 1.53 ± 0.08% methionine and less than 0.25% cystine." This protein was used in place of casein in the diet in order to reduce the methionine content.[7] The effect of adding various supplements to this diet is summarized in table 2. Growth was not stimulated by dimethylaminoethanol unless methionine was also added. Perosis was not produced in chicks on the basal diet, but appeared in chicks which received the basal diet plus methionine. The results indicate that methionine was the limiting dietary factor when diet 2 was fed to chicks, but growth on diet 2 was not rapid even when this diet was supplemented with both methionine and choline.

TABLE 2

Effect on growth and perosis in chicks produced by adding various supplements to a diet deficient in choline and methionine (diet 2).

ADDITION TO 100 GM. BASAL DIET 2	INCIDENCE OF PEROSIS AT 21 days	INCIDENCE OF PEROSIS AT 28 days	GAIN IN 28 DAYS
	%	%	*gm.*
None	0	0	27
0.2 gm. dimethylaminoethanol	0	0	20
0.2 gm. choline	0	0	37
0.6 gm. methionine	60	60	108
0.6 gm. methionine plus 0.2 gm. dimethylaminoethanol	0	0	127
0.6 gm. methionine plus 0.2 gm. choline	0	0	139

DISCUSSION

It is apparent that differences exist between the rat and the chick with regard to the utilization of certain precursors of choline. It has been observed with rats that ethanolamine (Stetten, '41) may function as a "methyl acceptor" for the biological synthesis of choline, and that ethanolamine may be formed from glycine (Stetten, '41) or serine (Stetten, '42). Methionine (du Vigneaud, '42–'43) or betaine (Simmonds et al., '44) may donate methyl groups to aminoethanol in the biological synthesis of choline in the rat. In the case of chicks fed certain purified diets, it has been found that choline deficiency is not alleviated by supplements of betaine, methionine or methionine plus aminoethanol (Jukes, '41; Jukes and Welch, '42; Almquist and Grau, '44). However it was found by McGinnis and co-workers ('44) that betaine or methionine would relieve choline deficiency in chicks fed certain diets of natural foods.

[7] Diet 2 was found to give a response corresponding to about 0.010% of choline and "Alpha protein" to about 0.009% of choline when assayed by the Neurospora method.

It has been shown (Horowitz and Beadle, '44) that mutant no. 34486 of Neurospora crassa is unable to grow in a purified culture medium, but will grow if a few micrograms of choline are added. These workers also observed that mutant no. 34486 is unable to use betaine or aminoethanol as substitutes for choline and that methionine has very little effect. Recently it has been found (Jukes, Dornbush and Oleson, '45) that the growth-stimulating power of betaine, aminoethanol and methionine for this mutant was not increased by using combinations of aminoethanol and betaine, or aminoethanol and methionine. It may be postulated that mutant no. 34486 has lost the ability, which the wild strain of Neurospora crassa may be presumed to possess, to methylate aminoethanol.

It has also been observed that the chick (Jukes and Oleson, '45), like mutant no. 34486, can utilize dimethylaminoethanol in place of choline (Jukes and Dornbush, '45). These various observations lead to the postulation that dimethylaminoethanol represents an intermediate step in the formation of choline by the "methylation" of aminoethanol.

The results in table 1 indicate that monomethylaminoethanol is utilized by the chick similarly to dimethylaminoethanol but less efficiently, as though monomethylaminoethanol were possibly a precursor of dimethylaminoethanol. A preceding step may be the formation of monomethylaminoethanol from aminoethanol and a "methyl donator". This step is apparently not accomplished by the chick as judged by the ineffectiveness of a supplement of methionine plus aminoethanol (Jukes, '41).

A response differing from those observed with the rat and the chick has been reported for a strain of Type III pneumococcus (Badger, '44). This organism can utilize ethanolamine, which it appears to be unable to synthesize, in place of choline in a basal medium containing methionine. A large number of substituted ethanolamines were also active.

SUMMARY

1. Monomethylaminoethanol and dimethylaminoethanol were found to prevent perosis in chicks when added to a purified diet which was deficient in choline. Growth was promoted by dimethylaminoethanol but not by monomethylaminoethanol.

2. The addition of methionine to the diet did not stimulate growth but growth was increased when methionine was added to the basal diet plus monomethylaminoethanol or to the basal diet plus dimethylaminoethanol.

3. Experiments were also made with a basal diet deficient in both methionine and choline. Growth was not stimulated by dimethylaminoethanol unless methionine was also added. Chicks receiving the basal diet did not develop perosis, but perosis developed in chicks receiving the basal diet plus methionine.

4. These observations are compared with certain results which have been reported in experiments with the rat and with the "cholineless" mutant no. 34486 of Neurospora crassa.

LITERATURE CITED

ALMQUIST, H. J., AND C. R. GRAU 1944 Interrelation of methionine, choline, betaine and arsenocholine in the chick. J. Nutrition, vol. 27, p. 263.

BADGER, E. 1944 The structural specificity of choline for the growth of type III pneumococcus. J. Biol. Chem., vol. 153, p. 183.

DU VIGNEAUD, V. 1942–1943 The significance of labile methyl groups in the diet and their relation to transmethylation. The Harvey Lectures Series 38, pp. 39–62.

GRAU, C. R., AND H. J. ALMQUIST 1943 The utilization of the sulfur amino acids by the chick. J. Nutrition, vol. 26, p. 631.

HOROWITZ, N. H., AND G. W. BEADLE 1943 A microbiological method for the determination of choline by use of a mutant of Neurospora. J. Biol. Chem., vol. 150, p. 325.

JUKES, T. H. 1941 The effect of certain organic compounds and other dietary supplements on perosis. J. Nutrition, vol. 22, p. 315.

JUKES, T. H., AND A. C. DORNBUSH 1945 Growth stimulation of Neuropsora cholineless mutant by dimethylaminoethanol. Proc. Soc. Exp. Biol. and Med., vol. 58, p. 142.

JUKES, T. H., A. C. DORNBUSH AND J. J. OLESON 1945 Unpublished observations.

JUKES, T. H., AND J. J. OLESON 1945 Choline-like nutritional effect of dimethylaminoethanol. J. Biol. Chem., vol. 157, p. 149.

JUKES, T. H., AND A. D. WELCH 1942 The effect of certain analogues of choline on perosis. J. Biol. Chem., vol. 146, p. 19.

MCGINNIS, J., L. C. NORRIS AND G. F. HEUSER 1944 Influence of diet on chick growth-promoting and antiperotic properties of betaine, methionine and choline. Proc. Soc. Exp. Biol. and Med., vol. 56, p. 197.

SIMMONDS, S., MILDRED COHN, J. F. CHANDLER AND V. DU VIGNEAUD 1944 Further studies of transmethylation with deuterium. Abstracts of Papers, 108th Meeting, American Chemical Society, p. 49B.

STETTEN, D., JR. 1941 Biological relationships of choline, ethanolamine, and related compounds. J. Biol. Chem., vol. 140, p. 143.

———— 1942 The fate of dietary serine in the body of the rat. J. Biol. Chem., vol. 144, p. 501.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

V. THE EFFECT OF COPPER ON EXTRA DIETARY ASCORBIC ACID [1]

MELVIN HOCHBERG, DANIEL MELNICK AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Certain food products may contain appreciable quantities of both ascorbic acid and copper. The best example is liver, reported to contain approximately 35 mg. of the vitamin and 2 mg. of copper per 100 gm. (Waisman and Elvehjem, '41). There are also pharmaceutical preparations that offer mixtures of vitamins and minerals including ascorbic acid and copper. A necessary precaution in assaying such products is to minimize the period of contact between the copper and ascorbic acid during the extraction of the material. The incompatability of copper and ascorbic acid in the presence of oxygen is well recognized. This raises the question of what happens in the digestive tract when prolonged contact between the vitamin and copper occurs.

Stotz, Harrer, Schultze and King ('38) reported that ascorbic acid oxidase preparations have no more catalytic activity than a solution of non-specific protein containing an equivalent amount of copper. A mixture of egg albumen and traces of copper was shown by these investigators to simulate all the observed properties of their ascorbic acid oxidase preparation. Some doubt is cast on this interpretation by the thermolability and optical specificity of ascorbic acid oxidase, properties which strongly suggest a specific protein as the catalyst. Nevertheless, there are indications in the literature (Ramasarma and associates, '40; Lovett-Janison and Nelson, '40) that this enzyme may be a copper-protein complex.

Mapson ('41) has employed the catalytic action of copper (approximately 4 μg. per milliliter) on the oxidation of ascorbic acid (100 μg. per milliliter) in relatively pure systems for evaluating the influence of various agents on stabilizing the vitamin. In the absence of a stabilizer, destruction occurred in solutions when the copper concentration ex-

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

ceeded 0.0015 μg. per milliliter. This small amount of copper was rarely obtainable unless special precautions were taken to eliminate copper as a contaminant of the reagents employed. No attempts were made by Mapson to measure dehydroascorbic acid since the latter was regarded as an oxidation product. However, from the practical standpoint, such data would have been important, since dehydroascorbic acid is biologically active. Fortunately, an earlier investigation by Barron and associates ('36) included such measurements. When the catalytic oxidation of ascorbic acid due to the presence of copper was performed in acid solutions (pH 5.0 or less), the oxidized form could be completely reduced to ascorbic acid by hydrogen sulfide, indicating it to be dehydroascorbic acid. Above pH 5.0 the amount of ascorbic acid recovered gradually decreased, until at pH 7.6 only 4% of the oxidized form could be reconverted to reduced ascorbic acid, the oxidation having gone beyond the dehydro stage.

In the present study of the influence of concomitant ingestion of copper on the availability of ascorbic acid in man, the photometric method of Hochberg, Melnick and Oser ('43) was employed for the determination of both the reduced and dehydro forms of the vitamin. The basal diet and test subjects were the same as those previously employed (Melnick, Hochberg and Oser, '45).

EXPERIMENTAL PART

In the preliminary in vitro studies measurements were made of both reduced and dehydroascorbic acid. However, in the case of the urine samples obtained from the test subjects, only total ascorbic acid was determined. In a previous investigation (Hochberg, Melnick and Oser, '45) dealing with the influence of dietary ascorbic acid oxidase on the availability of the vitamin, the urinary figures were partitioned into reduced and dehydroascorbic acid. No differences were observed in the ratio of one to the other during control and test periods despite the demonstrated effectiveness of the oxidase in converting reduced to dehydroascorbic acid prior to absorption. These findings and those reported by Johnson and Zilva ('34) indicated that nothing was to be gained by partitioning urinary ascorbic acid. The latter investigators found no difference in the form and amount of ascorbic acid excreted in the urine following test doses of reduced or dehydroascorbic acid. More recently Berryman and associates ('44) concluded that, although approximately 18% of the total ascorbic acid excreted following dosage with the reduced form of the vitamin is dehydroascorbic acid, this fraction remains relatively constant so that the measurement of either

reduced or total ascorbic acid is satisfactory in short (6-hour) experiments dealing with test dose responses. In the present studies, involving analyses of 24-hour urine samples during the collection of which opportunities exist for the partial and variable conversion of reduced into dehydroascorbic acid (Hochberg, Melnick and Oser, '43), it was considered advisable to measure total rather than only reduced ascorbic acid.

TABLE 1

In vitro tests of the incompatability of copper and ascorbic acid in the basal diet[1] before and after heat inactivation of the enzymes

EXPERIMENT		ASCORBIC ACID FOUND IN DIET		
No.	Condition[2]	Reduced	Dehydro	Total
		mg	*mg*	*mg*
I	Blanched,[3] homogenized basal diet	101	13	114
II	I incubated 6 hrs. at 37°C.	65	27	92
III	I + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	34	38	72
IV	I + 200 mg. of ascorbic acid, incubated 6 hrs. at 37°C.	240	39	279
V	I + 200 mg. of ascorbic acid + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	230	34	264
VI	Unblanched, homogenized basal diet	0	112	112
VII	VI incubated 6 hrs. at 37°C.	1	44	45
VIII	VI + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	1	35	36
IX	VI + 200 mg. of ascorbic acid, incubated 6 hrs. at 37°C.	128	78	206
X	VI + 200 mg. of ascorbic acid + 7 mg. copper,[4] incubated 6 hrs. at 37°C.	134	64	198

[1] 6.5 mg. of copper were found to be naturally present in the diet.

[2] The homogenized mixtures were incubated at their natural pH 5.5 in open beakers and stirred every half hour.

[3] The solid items in the ration were subdivided into small particles (about 1 cu. in.) and dropped immediately into boiling milk (previously deaerated) under an atmosphere of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized under nitrogen.

[4] Copper sulfate was used.

In vitro tests

In table 1 are presented the results of in vitro experiments on the incompatability of copper and ascorbic acid. Two series of tests were conducted, both on the homogenized basal diet.[2] For one series, the foods [3] were first blanched in order to inactivate all enzymes present;

[2] The ration included bread, butter, steak, milk, eggs, sugar, orange, banana, apple, lettuce, tomato and cooked carrots, beets and potatoes.

[3] The solid items in the ration were subdivided in small particles (about 1 cu. in.) and dropped immediately into boiling milk previously deaerated by a stream of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized in a Waring Blendor under nitrogen. In the case of the unblanched diet, the homogenization was carried out under air.

for the other, the unblanched homogenized ration was employed. Aliquots of the dietary mixtures were then maintained for a period of 6 hours at 37°C. at their natural pH of 5.5 in open beakers and gently stirred every ½ hour. The experiments were repeated but this time 7 mg. of copper, as copper sulfate, were added to the daily ration.[4] To other aliquots of the dietary, the equivalent of 200 mg. of ascorbic acid was added, with and without the additional 7 mg. of copper.

The results of the tests indicate that during the incubation of a dietary mixture in which all enzymes have been heat-inactivated there was a small but appreciable loss of ascorbic acid. That this was due to oxidation may be inferred from the fact that the dehydroascorbic acid value had increased. In the presence of added copper this oxidative loss, involving conversion of reduced to dehydroascorbic acid, progressed somewhat further. That these losses in the ascorbic acid appear to be absolute, was shown by the experiments in which added ascorbic acid was quantitatively recovered in the reduced state from the dietary mixture (compare experiments IV and V with experiments II and III, respectively). These tests on the blanched homogenized basal diet are significant since the effect of copper per se was measured; no active enzymes or native protein were present to complicate the interpretation.

Simple homogenization of the unblanched homogenized basal diet resulted in marked oxidation of the ascorbic acid, practically all of it going to but not beyond the dehydroascorbic stage. However, during the period of incubation of the dietary mixture at 37°C. fully 60% of the dehydroascorbic acid was further oxidized. In the presence of added copper there was a small additional loss of dehydroascorbic acid. Extra ascorbic acid added to the mixture was mostly oxidized to and some even beyond the dehydroascorbic acid stage. Additional copper caused no more marked destruction of the ascorbic acid. In this series of experiments the decreases in ascorbic acid content of the dietary mixtures were due principally to the action of ascorbic acid oxidase. This enzyme is believed (Green, '41) to catalyze the oxidation of ascorbic acid in an absolute manner, the rate of oxidation being independent of the concentration of the vitamin. The present results on the fate of extra ascorbic acid added to the incubated dietary mixtures confirm this hypothesis. The findings with added ascorbic acid and added copper, however, fail to support the suggestion advanced by Stotz and associates ('38) that ascorbic acid oxidase is a non-specific protein-copper complex. If this were the case, an increase in the oxidative destruction of ascorbic

[4] 6.5 mg. of copper were found by analysis (McFarlane, '32) to be naturally present in the 2500 gm. of dietary mixture; this was equivalent to 2.6 µg. of copper per ml. of mash.

acid should have occurred when additional quantities of copper were added to the unblanched homogenized dietary mixture containing appreciable amounts of native protein. This was not noted.

In the present series of experiments the dietary mash contained approximately 2.6 μg. of copper per milliliter. The addition of the 7 mg. of extra copper to the whole day's ration increased the copper concentration to approximately 5.4 μg. of copper per milliliter. Despite the fact that this concentration is greater than the 4.0 μg. of copper per milliliter, employed by Mapson ('41) in his experiments dealing with factors affecting the stability of ascorbic acid in pure systems, much smaller losses of ascorbic acid were observed. It is to be noted that Mapson kept the test solutions at 100°C. though for a period only one-twelfth of that employed in the present investigation. It may very well be that in the pure systems employed by Mapson much greater destruction of the vitamin occurred primarily because of the absence of natural protective agents. It is known that in natural extracts there are present various substances, such as glutathione and other sulfhydryl compounds, which are capable of protecting the vitamin by their preferential oxidation. Such factors were undoubtedly operative in the present study.

In vivo test

The physiological availability study involved measurements of the extra urinary excretion of ascorbic acid by human subjects receiving a constant basal diet and taking a 200-mg. test dose of extra ascorbic acid in pure solution, at one period alone, and the other followed by a solution of copper sulfate. The amount of copper in the test dose was 7.0 mg. The ratio of added ascorbic acid to added copper in both the in vitro and in vivo tests (200: 7) is the same as the ratio, but about seven times the amount, of the minimum daily requirements for these factors.

The results of the availability studies are summarized in table 2. These indicate that no destruction of ascorbic acid occurred in the digestive system as the result of taking copper along with the vitamin in the doses indicated. These results were in good agreement with the data obtained in the in vitro test. It may be emphasized that these studies relate to the effect of copper ingestion on the fate of extra dietary ascorbic acid; whether prolonged ingestion of copper would depress the basal excretion (i.e., of vitamin from the diet itself) remains to be demonstrated.

TABLE 2

Availability of ascorbic acid in presence of copper.
(All values in milligrams per 24 hours.)

SUBJECT	CONTROL PERIOD: NO COPPER, 200 MG. ASCORBIC ACID			TEST PERIOD: 7.0 MG. COPPER, 200 MG. ASCORBIC ACID		
	Basal excretion	After test dose	Per cent of test dose excreted	Basal excretion	After test dose	Per cent of test dose excreted
J.C.	57	183	63	23	130	54
E.M.	41	159	59	17	130	57
D.M.	47	131	42	50	150	50
M.H.	70	162	46	24	152	64
Average	54	159	53	29	141	56

Availability of ascorbic acid in presence of added copper $= \frac{56}{53} \times 100 = 106\%$.

SUMMARY

The addition of 7 mg. of copper to a homogenized mixture of a day's ration and incubation of this mixture for a period of 6 hours at 37°C. resulted in a small but real destruction of the ascorbic acid present. Since no more vitamin was destroyed in the presence of added ascorbic acid (200 mg.), this destruction is regarded as absolute. The factor primarily responsible for the marked loss of ascorbic acid in a homogenized dietary mixture is ascorbic acid oxidase. The addition of extra copper to such a mash (unblanched) failed to produce markedly greater destruction of vitamin C. This would seem to indicate that ascorbic acid oxidase is a specific enzyme and not simply a loose complex of copper with protein of unspecific nature. Human availability studies have indicated that in the digestive tract ascorbic acid and copper in the quantities taken, 200 mg. and 7 mg., respectively, are not incompatible.

LITERATURE CITED

BARRON, E. S. G., R. H. DEMEIO AND F. KLEMPERER 1936 Studies on biological oxidations. V. Copper and hemochromogens as catalysts for the oxidation of ascorbic acid, the mechanism of the oxidation. J. Biol. Chem., vol. 112, p. 625.

BERRYMAN, G. H., C. E. FRENCH, H. A. HARPER AND H. POLLACK 1944 Response to the intravenous injection of ascorbic acid as indicated by the urinary excretion of the total and reduced forms. J. Nutrition, vol. 27, p. 309.

GREEN, D. E. 1941 Mechanism of biological oxidations. Cambridge University Press, London, England.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1943 Photometric determination of reduced and total ascorbic acid. Ind. Eng. Chem., Anal. Ed., vol. 15, p. 182.

——— 1945 Physiological availability of the vitamins. III. The effect of dietary ascorbic acid oxidase. J. Nutrition, vol. 30, p. 193.

JOHNSON, S. W., AND S. S. ZILVA 1934 The urinary excretion of ascorbic acid and dehydroascorbic acids in man. Biochem. J., vol. 28, p. 1393.

LOVETT-JANISON, P. L., AND J. M. NELSON 1940 Ascorbic acid oxidase from summer crookneck squash (C. pepo condensa). J. Am. Chem. Soc., vol. 62, p. 1409.

McFARLANE, W. D. 1932 Application of the sodium diethyldithiocarbamate reaction to the micro-colorimetric determination of copper in organic substances. Biochem. J., vol. 26, p. 1022.

MAPSON, L. W. 1941 The influence of halides on the oxidation of ascorbic acid. Biochem. J., vol. 35, p. 1332.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

RAMASARMA, G. B., N. C. DATTA AND N. S. DOCTOR 1940 Ascorbic acid oxidase. Enzymologia, vol. 8, p. 108.

STOTZ. E., C. J. HARRER, M. O. SCHULTZE AND C. G. KING 1938 The oxidation of ascorbic acid in the presence of guinea pig liver. J. Biol. Chem., vol. 122, p. 407.

WAISMAN, H. A., AND C. A. ELVEHJEM 1941 The vitamin content of meat. Burgess Publishing Co., Minneapolis.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

VI. THE EFFECT OF ADSORBENTS ON THIAMINE [1]

DANIEL MELNICK, MELVIN HOCHBERG AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

The ingestion of various adsorbents for one therapeutic reason or another is not an uncommon practice. However, some of these substances have the capacity to adsorb the B-vitamins and indeed have been employed for the concentration and isolation of these vitamins from natural sources.

Prior to the synthesis of thiamine, the International Standard for vitamin B_1 was a fuller's earth adsorbate of an extract of rice polish. This particular preparation was subsequently shown by Keresztesy and Sampson ('37) to be unsatisfactory as a reference standard. In rat curative tests they found that a quinine sulfate eluate of the clay was fully twice as effective as the direct feeding of the adsorbate to the animal. Apparently the adsorbate contained twice as much thiamine as indicated by the standard assay, but the animal organism could elute only one-half. Despite these findings some pharmaceutical preparations still include fuller's earth adsorbates of the B vitamins.

In 1940 Robinson, Melnick and Field reported that almost without exception patients with peptic ulcers receiving antacid medication excreted very small amounts of thiamine in the urine before and after the taking of a test dose of the vitamin. It was subsequently demonstrated (Melnick, Robinson and Field, '41) by in vitro and in vivo experiments that the antacid medication and not the thiamine content of the diet was at fault. The creation of an alkaline reaction in the gastrointestinal tract favored greater destruction of thiamine, while adsorbents, particularly magnesium trisilicate, caused a loss of thiamine to the organism by rendering the vitamin unavailable for absorption.

Adsorbents have been recommended for the treatment of certain intestinal disorders. Kaolin has long been employed for the control of

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th Meeting of the American Chemical Society, New York, N. Y. The expenses of this study were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

diarrhea when the agent responsible for the condition is capable of being adsorbed, for example, in cases of food poisoning or infection with gastroenterotoxic organisms. Since chronic diarrhea can also be responsible for conditioned malnutrition (Jolliffe, '43), certain pharmaceutical products consisting of mixtures of kaolin and vitamins, particularly those of the B-complex, have been recommended for the treatment of the diarrhea and at the same time for effecting restoration of the vitamin content of the tissues. However, adsorption is not a specific phenomenon. Adsorbing agents, taken orally, can bind nutrients and enzymes, as well as toxic substances present in the gastrointestinal tract. Thus, the possibility exists that the presence of the kaolin may render unavailable not only the vitamins taken with the adsorbent but also nutrients in the dietary which are capable of being adsorbed. This action of adsorbents may be likened to that of mineral oil in reducing the availability of fat-soluble vitamins because of their preferential solubility in the unabsorbable oil and their subsequent excretion in the stools.

In the present study the influence of the concomitant ingestion of fuller's earth and of kaolin on the availability of thiamine to man was investigated. Use was made of the bioassay technic described in a previous paper (Melnick, Hochberg and Oser, '45). The vitamin intake was determined by the thiochrome procedure (Hennessy, '41) and urinary excretion by the colorimetric method (Hochberg and Melnick, '44).

Thiamine was selected as the vitamin for study since in vitro tests demonstrated that it was more readily adsorbed and more firmly retained by the adsorbents than either of the other two major vitamins of the B complex, riboflavin and niacin, for which the assay technic is applicable.

EXPERIMENTAL PART

In vitro tests

That both fuller's earth and kaolin can readily adsorb thiamine from aqueous solution is indicated by the data in table 1. For each experiment a solution containing 5 mg. of thiamine was used. The pH of the suspension was 4.5 and contact between vitamin and adsorbent was for a period of 6 hours at 37°C. The results suggest that fuller's earth may have a greater affinity than kaolin for thiamine.

In vivo tests

Two commercially available products were employed as the test materials in the human availability studies: (a) a B-complex gelatin cap-

sule, containing fuller's earth; and (b) a pharmaceutical preparation of pure kaolin recommended for the control of diarrhea. The adsorbents were the same as those employed in the in vitro tests described above. Each assay involved comparison of the extra urinary excretion of thiamine by the test subjects receiving a uniform, adequate basal diet and taking a test dose (5.0 mg.) of thiamine during the control period, as a pure solution alone; during the test period, along with the adsorbent.

The results of the availability study in which five of the vitamin capsules were taken postprandially by each of the test subjects are presented in table 2. Each vitamin capsule contained 1000 μg. of thiamine

TABLE 1

In vitro adsorption of thiamine from pure aqueous solutions.[1]

ADSORBENT	QUANTITY	THIAMINE ADSORBED
	gm.	%
	0.25	99
Fuller's earth	0.50	100
	2.00	100
	0.5	54
Kaolin	1.0	73
	100.0	100

[1] In each experiment the adsorbent was added to an acetate-buffered (pH 4.5) solution containing 5 mg. of thiamine. Contact between vitamin and adsorbent was at 37°C. for a period of 6 hours. The total volume was 30 ml. in every case except in the last instance where the 100 gm. of kaolin were added to 1500 ml. of solution.

TABLE 2

Availability to man of thiamine in B-complex capsule containing fuller's earth.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 5.0 MG. THIAMINE			TEST PERIOD: 5 CAPSULE[1] DOSE, 5.0 MG. THIAMINE		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	0.20	1.08	18	0.24	0.41	3
E.M.	0.23	1.00	15	0.27	1.08	16
D.M.	0.30	1.35	21	0.29	0.58	6
M.H.	0.26	1.50	25	0.21	0.59	8
H.H.	0.25	1.39	23	0.23	0.48	5
Average[2]	0.25	1.33	22	0.24	0.52	6

Availability of thiamine in capsules $= \frac{6}{22} \times 100 = 27\%$.

[1] Each containing 333 mg. of fuller's earth as a riboflavin adsorbate.
[2] Omitting subject E.M.

and 500 of riboflavin, the latter adsorbed to 333 mg. of fuller's earth.[2] However, the available thiamine content of these capsules was found to be only 27% of the total, as indicated by the four subjects showing diminished excretion of the test dose.[3]

It is of interest to note that one subject was able to elute the thiamine completely from the fuller's earth adsorbate. The test was repeated with this individual and essentially the same results were obtained. This variability in the responses of the human subjects finds its counterpart in the variability in the responses of rats in the old biological assay procedure when the International Standard was employed as the reference material.

In order to ascertain whether the apparent low availability of thiamine was due simply to a slow rate of absorption of the vitamin, evidenced by a delay in its excretion, analyses were conducted on the urine samples collected during the second 24-hour period following dosage. The "carry-over" values for each period were found to be small, in each case about one-sixth of the extra urinary excretion figures for the first 24 hours following dosage. Since these carry-over values bore the same relationship to each other as the figures for the first 24-hour samples, delayed absorption could not have been responsible for the low thiamine availability.[4]

A similar study was conducted to determine the influence of the concomitant ingestion of kaolin on the availability of thiamine. In the control of diarrhea it is the practice to give kaolin in doses of from 50 to 100 gm. at 3-hour intervals (Goodman and Gilman, '41). Smaller doses of 5 to 10 gm. are frequently taken daily over long periods of time to

[2] Thus, the thiamine was incorporated in the mix prior to encapsulation as the free crystalline material. However, during the disintegration of the tablet in vivo adsorption of the thiamine undoubtedly occurs due to the favorable pH of the gastric contents. Indeed, even when this product was assayed chemically, interference in the extraction of the thiamine was encountered. A value of 688 µg. of thiamine per capsule was obtained by the standard thiochrome procedure (U. S. Pharmacopoeia XII, '42). Only after exhaustive strong acid (2N HCl) extraction could the claimed quantity of thiamine (1000 µg.) be found present. This led to the suspicion that the vitamin in this capsule would only be partly available to man.

[3] Similar studies indicated that only 79% of the riboflavin present in these capsules was available. In vitro tests also indicated that the riboflavin could be more easily extracted from the adsorbent than thiamine.

[4] In other related studies conducted in these laboratories analyses were conducted on the urine samples collected during the second as well as the first 24-hour period following dosage. In no case did the carry-over values (second 24-hour excretions) alter the interpretation of the data based solely on the figures obtained for the first 24-hour samples. If vitamin absorption from the gastrointestinal tract is to be adequate, it should occur well within the first 24 hours after dosage; otherwise the vitamins are excreted in the stools and thereby lost to the organism.

regulate bowel action. In the present study a single dose of 100 gm. of kaolin in aqueous suspension was taken postprandially by each subject. The test dose of thiamine followed immediately after the kaolin suspension had been taken. The thiamine was dissolved in milk rather than in water as had been customary. This was intended to provide a longer period of contact in the stomach between the kaolin and the thiamine, thereby favoring adsorption of a greater portion of the dose. Furthermore, the design of the present experiment more nearly duplicated conditions in practice with respect to the action of the adsorbent on dietary thiamine.

TABLE 3

Availability of thiamine in presence of kaolin.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 5.0 MG. THIAMINE[1]			TEST PERIOD: 100 GM. KAOLIN FOLLOWED BY 5.0 MG. THIAMINE IN SOLUTION		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	0.20	1.45	25	0.20	1.56	27
E.M.	0.23	0.89	13	0.24	1.48	25
D.M.	0.30	1.13	17	0.29	1.40	22
M.H.	0.26	1.17	18	0.23	1.58	27
H.H.	0.24	1.20	19	0.26	1.23	19
Average	0.25	1.17	18	0.24	1.45	24

Availability of thiamine in presence of kaolin $= \frac{24}{18} \times 100 = 133\%$.

[1] Thiamine taken dissolved in milk.

The results of the urinary excretion tests following dosage with kaolin and extra thiamine are presented in table 3. These indicate that kaolin, unlike fuller's earth, does not diminish the availability of thiamine. The percentage excretion of the test dose following the taking of the vitamin along with kaolin was not less than that noted when the pure solution of thiamine was taken alone. In vitro tests (see table 1) have indicated that kaolin adsorbs the vitamin quite readily and the pH of the gastric contents is optimal for this adsorption. Apparently, at the more alkaline pH of the small intestine, the thiamine is effectively eluted from the kaolin adsorbate. The greater-than theoretical availability of the vitamin, noted in three of the five subjects, is real and not due to experimental error. In some experiments which have been reported (Oser, Melnick and Hochberg, '45) and in others which are in progress in our laboratories, it has become evident that thiamine shows

a variable degree of stability in the gastrointestinal tract prior to absorption and that the vitamin may be protected by the concomitant ingestion of various materials.

SUMMARY AND CONCLUSIONS

Certain substances, capable of adsorbing the B vitamins, are taken regularly and in appreciable quantities over long periods of time for the treatment of various clinical disorders. Human availability studies have been conducted to determine the influence of the concomitant ingestion of two such adsorbents, fuller's earth and kaolin, on the availability of thiamine. These materials were shown to adsorb the vitamin very readily from aqueous solution. Whereas the availability of thiamine was markedly reduced by the fuller's earth, no interference occurred when kaolin was taken along with thiamine. Indeed, the latter adsorbent appeared to protect the vitamin during its passage through the gastrointestinal tract so that a greater-than-theoretical value for available thiamine was obtained. In vitro tests have not proved satisfactory for predicting the influence of an adsorbent on vitamin availability. The present experiments cast doubt upon the wisdom of taking continuous large doses of adsorbing agents, unless precautions are taken to insure sufficient vitamin intake.

LITERATURE CITED

GOODMAN, L., AND A. GILMAN 1941 The Pharmacological Basis of Therapeutics. Macmillan Co., New York.

HENNESSY, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal. Ed., vol. 13, p. 216.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1944 A simple modification of the colorimetric method for routine thiamine clearance tests. J. Biol. Chem., vol. 156, p. 53.

JOLLIFFE, N. 1943 Conditioned malnutrition. J. Am. Med. Assn., vol. 122, p. 299.

KERESZTESY, J. C., AND W. L. SAMPSON 1937 Utilization of vitamin B_1 from Fuller's earth adsorbates. Proc. Soc. Exp. Biol. Med., vol. 36, p. 686.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

MELNICK, D., W. D. ROBINSON AND H. FIELD, JR. 1941 Fate of thiamine in the digestive secretions. J. Biol. Chem., vol. 138, p. 49.

OSER, B. L., D. MELNICK AND M. HOCHBERG 1945 Physiological availability of the vitamins. VII. Study of methods for determining the availability of the vitamins in pharmaceutical products. Ind. Eng. Chem., Anal. Ed., (in press).

ROBINSON, W. D., D. MELNICK AND H. FIELD, JR. 1940 Urinary excretion of thiamine in clinical cases and the value of such analyses in the diagnosis of thiamine deficiency. J. Clin. Invest., vol. 19, p. 399.

U. S. PHARMACOPOEIA XII. First bound supplement 1943 Thiochrome assay for thiamine hydrochloride. Pp. 82–84.

EFFECT OF SOYBEAN PHOSPHATIDES ON VITAMIN A METABOLISM

CHARLES A. SLANETZ AND ALBERT SCHARF
Department of Animal Care, Columbia University, New York

TWO FIGURES

(Received for publication March 20, 1945)

We have previously reported that commercial soybean lecithin contains an unknown factor which influences carotene and vitamin A utilization in the rat (Slanetz and Scharf, '43). As pointed out in this report, our diet differed from that of other investigators insofar as we substituted the synthetic members of the B complex for yeast as a supply of the B vitamins known to be essential for normal growth in the rat.

Patrick and Morgan ('43) confirmed our findings in the chick and also demonstrated that the unknown factor may be extracted from yeast by suitable solvents. Earlier, Polskin ('40) had reported about the effect of lecithin on the utilization of vitamin A in the chick and found better growth response and an increased liver storage of vitamin A when lecithin was added to the diet. No difference in total liver lipids could be noted between the controls and the lecithin-treated animals.

Adlersberg and Sobotka ('43) reported elevated vitamin A blood levels in man after ingestion of soybean lecithin.

Jensen, Hickman and Harris ('43), however, questioned our findings because our soybean lecithin preparation contained vitamin E and they referred the observed effect to the presence of this vitamin. However, the diet in their experiment contained 8% yeast and, therefore, their experimental conditions were not comparable to ours.

In a recent report (Scharf and Slanetz, '44) we proved that vitamin E is not the factor responsible for our observations and while this report was in the printer's hands Patrick and Morgan ('44) published their experiments which also proved that vitamin E was not responsible for the reported action of soybean lecithin. As a matter of fact, Patrick and Morgan as well as we, found indications of vitamin E deficiency in the animals in spite of sufficient amounts of vitamin E in the diet.

In our previous report (Slanetz and Scharf, '43) we had mentioned the well known relation between soybean cephalin, contained in our commercial soybean lecithin, and vitamin E concerning its antioxidant effectiveness. Patrick and Morgan ('44) also seemed to be inclined to explain the observed effects as the stabilizing influence of soybean cephalin on vitamin E and indirectly on vitamin A.

Since our previous work (Slanetz and Scharf, '43) indicated that soybean lecithin influences utilization of vitamin A when borderline amounts of vitamin A are fed to rats, we felt it advisable to study the effect of soya lecithin on vitamin A utilization when larger amounts of vitamin A are fed and its effect on storage of vitamin A in the liver. In this report we present our findings on the above as well as the results of our study on the influence of heating, halogenation, and other factors on the unknown factor in soybean lecithin.

EXPERIMENTAL

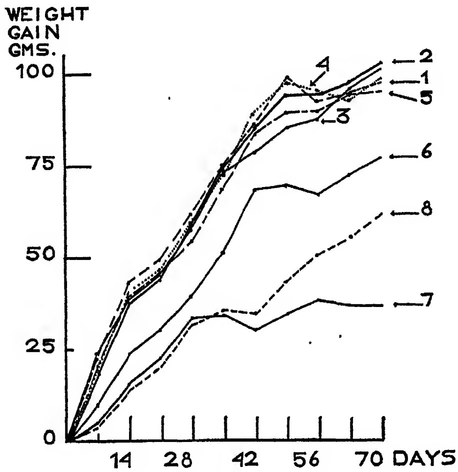
Female albino rats, Sherman strain, from our laboratory colony, weighing 90–110 gm. at the start of the experiment were kept in individual metal cages equipped with screen floors. The rats were weighed weekly. Food consumption was determined at intervals. The percentage composition of the basal diet was as follows: vitamin-free casein (Labco) 20; cerelose 72; Sure's no. 1 salts 4; corn oil 2; wheat germ oil 2; viosterol 0.002. The diet was supplemented with thiamine 2 mg., riboflavin 4 mg., pyridoxine 4 mg., nicotinic acid 100 mg., vitamin K 4 mg., inositol 1000 mg. and para-aminobenzoic acid 300 mg., per kilogram of diet. Synthetic calcium pantothenate (100 µg.) was given orally daily. Each rat was given orally 30 µg. of carotene daily. The purified diets and mixtures were freshly prepared every 2 weeks and stored at 4°C.

Forty-eight rats were distributed equally among eight groups and the groups fed as follows: basal diet only; and basal diet supplemented daily, respectively, with 3 mg. choline, 1% soybean lecithin, 1% heated soybean lecithin, 5% cholesterol, 1% iodized lecithin, 3% iodized lecithin, and 0.3% iodine in the form of potassium iodide. After 10 and 12 weeks on the diet several rats from each group were sacrificed and vitamin A determinations made on the blood and liver.

DISCUSSION

Commercial soybean lecithin is an extract of soybean lipids and contains about 30–35% soybean oil and about 65% soya phosphatides. There are also present small amounts of carbohydrates, phytosterols, and traces of other as yet unidentified substances. The soya phospha-

tides comprise soya lecithin, soya cephalin, and lipositol (inositol phosphatides) which are present in approximately equal amounts. Separation and purification of the various phosphatides and substances present in soybean lecithin has as yet not been successful and even the most purified fractions have been found to be contaminated to the extent of 10–20% with other substances. Commercial soybean lecithin



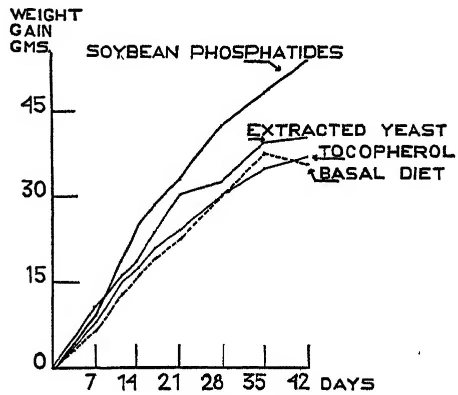


Figure 2

Figure 1

Fig. 1 Influence of various dietary supplements on the growth of rats. Group 1 was fed the basal diet only. The other groups received the basal diet supplemented as follows: group 2, 3 mg. choline daily; group 3, 1% soybean lecithin; group 4, 1% heated soybean lecithin; group 5, 5% cholesterol; group 6, 1% iodized lecithin; group 7, 3% iodized lecithin; group 8, 0.3% iodine in the form of potassium iodide.

Fig. 2 Influence of soybean phosphatides, ether-extracted yeast and tocopherol on weight response of rats.

is devoid of vitamin A but does contain about 1.2–1.4 mg. vitamin E per gram as we have reported before (Scharf and Slanetz, '44). We, as well as Patrick and Morgan, have proved that the vitamin E content of the soybean lecithin used is not responsible for the observed effects (fig. 2). Therefore, the presence of an unknown factor in commercial soybean lecithin that affects vitamin A storage in the liver is indicated by our findings.

Figure 1 shows that rats receiving an ample supply of vitamin A do not show any marked difference in growth response whether given soybean lecithin or not. However, there was a marked increase of vitamin A in the blood as well as a very striking elevation of the liver vitamin A when the commercial lecithin was fed (table 1). Figure 1 also shows that addition of choline did not influence rate of growth.

The presence or absence of choline in our diet did not seem to influence either blood or liver vitamin A, nor did cholesterol. No gross pathology was observed in the livers, indicating a sufficient supply of the lipotropic factors, choline and inositol, in our diet.

Patrick and Morgan ('43) reported that a deficiency of the unknown factor could be developed in chicks receiving yeast extracted by fat solvents as a source of the vitamin B complex. Rats receiving such extracted yeast and a diet as described by us in the same report showed also deficiency symptoms, though to a somewhat lesser degree than the controls.

TABLE 1

Vitamin A in blood plasma and liver of rats fed various diets.

GROUP NO.	DIET	VITAMIN A	
		Blood plasma	Liver
		I.U./100 ml.	*I.U./100 gm.*
1	Basal diet only	93	20
	Basal diet supplemented by:		
2	Choline daily (3 mg.)	88	20
3	1% soybean lecithin	150	350
4	1% soybean lecithin heated	120	200
5	5% cholesterol	99	20
6	1% iodized lecithin	57	20
7	3% iodized lecithin	27	20
8	0.3% iodine (KI)	111	30

As it is known that heating lecithin to higher temperatures or halogenation of lecithin does not interfere with its antioxidant effect in vitro, iodized soybean lecithin as well as soybean lecithin[1] heated to 110°C. for 1 hour was fed to the rats. The results show that the heating of the soybean lecithin did not destroy its effectiveness but diminished it. On a diet as described in our previous experiment (Scharf and Slanetz, '44) heated lecithin showed a lesser effect on growth than unheated lecithin.

The incorporation of 10% iodine in soybean lecithin[2] apparently destroyed the effectiveness of the unknown factor. However, the rats receiving iodine in form of iodized lecithin or potassium-iodide also showed impaired growth. These results require further investigation.

The results described above do not indicate that the reported effect is identical with the antioxidant effect of soybean cephalin on vitamin

[1] We are indebted to the American Lecithin Company for supply of commercial soybean lecithin.

[2] We are indebted to Dr. P. L. Julian and Mr. H. Iveson of the Glidden Co., Chicago, Ill., for the iodized lecithin

E. Polskin ('40) observed that chicks receiving lecithin and vitamin A and having an elevated vitamin A content of the liver lose much less of their vitamin A reserve in a given time when put on a vitamin A deficient diet than those that receive no lecithin. This, likewise, is hard to explain as an antioxidant effect. Further experiments may determine whether the observed effect is due to the antioxidant action of cephalin or whether an unknown nutritional factor plays a role.

SUMMARY

Liver storage and blood levels of vitamin A in the rat have been investigated as influenced by specific factors used as supplements in a purified basal diet containing synthetic B vitamins instead of yeast. Under the conditions of our experiment commercial soybean lecithin markedly influenced storage and blood levels of vitamin A in the rat. Iodine interfered with this effect while heating of soybean lecithin interfered but little. The presence of an unknown factor in commercial soybean lecithin is indicated.

LITERATURE CITED

ADLERSBERG, D., AND H. SOBOTKA 1943 Influence of lecithin feeding on fat and vitamin A absorption in man. J. Nutrition, vol. 25, p. 255.

JENSEN, J. L., C. D. HICKMAN AND P. L. HARRIS 1943 Effect of tocopherols and soybean phosphatides on utilization of carotene. Proc. Soc. Exp. Biol. and Med., vol. 54, p. 294.

PATRICK, H., AND C. L. MORGAN 1943 An unidentified nutrient required for proper utilization of dl-alpha- tocopherol by the chick. Science, vol. 98, p. 434.

———— 1944 Studies on the role of vitamin E in chick nutrition. Poultry Science, vol. 23, p. 525.

POLSKIN, L. J. 1940 Studies on the absorption and retention of vitamin A and carotene by the chicken on normal and low fat rations. A thesis submitted to Rutgers University, New Brunswick, N. J.

SLANETZ, C. A., AND A. SCHARF 1943 The effect of phosphatides on utilization of vitamin A and carotene. Proc. Soc. Exp. Biol. and Med., vol. 53, p. 17.

SCHARF, A., AND C. A. SLANETZ 1944 Effect of soybean phosphatides on utilization of the fat-soluble vitamins. Proc. Soc. Exp. Biol. and Med., vol. 57, p. 159.

NUTRITIVE VALUE OF DISTILLERS' DRIED SOLUBLES AS A SOURCE OF PROTEIN [1, 2]

C. W. HUGHES AND S. M. HAUGE
Purdue University Agricultural Experiment Station, Lafayette, Indiana

(Received for publication March 30, 1945)

Distillers' dried solubles has recently assumed importance as an ingredient in commercial feeds for poultry and farm animals. Although distillers' solubles was a waste product prior to 1939, the production of dried solubles has increased rapidly during recent years. This product has been shown to be a valuable vitamin supplement (Bauernfeind et al., '44); Boruff et al., '40; Synold et al., '43). Because of its high protein content it also comes into consideration as a protein supplement. However, its value as a protein supplement has not received extensive study. Sloan ('41) found that it could be used satisfactorily to supply approximately 12% of the total protein in poultry rations. Bauernfeind et al. ('44) suggest its use as a supplement to soybean meal since it contains 2.7% methionine. Joseph et al. ('42, '43) reported that the amino acid composition of dried solubles resembled that of yeast and found that both the protein content and palatability could be improved by growing yeast in the stillage. The nutritive value of yeast has been reviewed by Carter and Phillips ('44).

In the present investigation the nutritive value of distillers' dried solubles is evaluated by biological and chemical means.

EXPERIMENTAL

The dried solubles used in this study was derived from a mash containing 51% corn, 42.5% wheat, and 6.5% barley malt. The thin sillage was concentrated in multiple-effect evaporators under reduced pressure at temperatures ranging from 130° to 230°F. to a thick syrup of approximately 30% solids and then dried on drum driers. The protein content of the moisture free product was 37.14%. It contained proteins derived from these cereal grains and yeast.

[1] Journal Paper no. 187 of the Purdue University Agricultural Experiment Station.
[2] This investigation was supported in part by a grant from Joseph E. Seagram and Sons Inc., Louisville, Kentucky.

The nutritive value of dried solubles was investigated by means of biological tests with albino rats as the experimental animal. In each series of experiments hydrogenated vegetable oil was used to adjust the rations to the same fat content so that differences in energy values should not be a factor affecting food consumption. In addition to the mixed rations, vitamin supplements containing sufficient amounts of all vitamins for normal growth, independent of the vitamin content of the rations were fed 3 times weekly in separate containers. The vitamin B complex supplement contained 200 mg. riboflavin, 2 gm. nicotinic acid, 100 mg. pyridoxine hydrochloride, 100 mg. thiamine chloride, 2 gm. calcium pantothenate, 30 gm. choline chloride, 300 gm. "Labco" rice polish concentrate no. II, and 10 gm. sodium chloride per liter. The cod liver oil contained not less than 1500 U.S.P. units of vitamin A and 100 U.S.P. units of vitamin D per gram. The amounts fed were equivalent to 27 mg. of cod liver oil and ½ ml. of vitamin B complex supplement per day.

Distillers' dried solubles as a sole source of protein

The growth value of dried solubles as a sole source of protein was determined and compared with values for corn and casein. Since casein is known to produce optimum growth at a 15% protein level, all comparisons were made at this level. In this trial ten rats, five male and five female, of an average weight of 42 gm. were placed on experiment. They were fed ad libitum for a period of 6 weeks. A record was kept of the weekly weight and food consumption of the rats. The composition of the rations and the results are given in table 1. The proteins of dried solubles (lot 2) were found to be more deficient than the proteins of corn (lot 1). In fact the proteins of dried solubles were so deficient that they contributed little above maintenance requirements. In contrast to the response with casein (lot 4) it is evident that the proteins of both dried solubles and corn are critically deficient.

Distillers' dried solubles as a supplement to corn

Two series of experiments were conducted in which dried solubles was compared with casein as a supplement to corn. The supplements constituted one-third, one-half and two-thirds of the total protein (15%). Because the growth responses of all lots within each series were almost identical, only one group from each series is reported (lots 5 and 7). Since combinations of dried solubles and corn gave poorer growth responses than corn alone (lot 1), it is apparent that the proteins of dried

TABLE 1

"Distillers' dried solubles" as a sole source of protein and as a supplement to corn, and the effect of fortification with dried brewers yeast.

LOT	1	2	3	4	5	6	7	8	9	10	11	12	13
Ration Ingredients (%)													
Yellow corn	54.5				54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5
Corn gluten meal	23.6							11.8	11.8	11.8	11.8	5.9	
Dried solubles		44.7			29.8			14.9	11.9	8.9	7.5	11.2	14.9
Dried yeast			30.8			20.5			2.1	4.1	5.1	7.7	10.3
Casein				17.5			11.7						
Dextrin	16.4	50.3	61.4	74.5	11.8	19.3	27.9	14.1	14.8	15.6	16.0	15.7	15.5
Hydrogenated vegetable oil	2.5	2.0	4.8	5.0	0.9	2.7	2.9	1.7	1.9	2.1	2.1	2.0	1.8
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Per cent of total protein from													
Corn	100				33.4	33.4	33.4	66.7	66.7	66.7	66.7	50.0	33.4
Dried solubles		100			66.6			33.3	26.7	20.0	16.7	25.0	33.3
Dried yeast			100			66.6			6.6	13.3	16.6	25.0	33.3
Casein				100			66.6						
Rats — number	10	10	10	10	10	10	10	10	10	10	10	10	10
Average gain (gm.) — 6 wks.[1]	67	26	68	189	55	138	191	54	87	104	119	123	144
Feed efficiency	5.14	8.09	4.82	3.08	5.4	3.57	3.20	5.58	4.51	4.18	3.91	3.91	3.73

[1] The standard error of the *difference* between any two average gains is 8.0 gm. Consequently the difference necessary for significance is 16 gm. at the 5% level or 21 gm. at the 1% level.

solubles do not supplement those of corn. This is emphasized by comparison with lot 7.

The effect of fortification of distillers' dried solubles with dried brewers' yeast

Since Joseph et al. ('43) found that the protein content of stillage derived from corn mashes could be increased by the growth of yeast in the product, studies were made to determine the feasibility from a nutritional viewpoint of increasing the yeast protein content of distillers' dried solubles. To simulate a product containing more yeast protein, dried solubles was used in combination with dried brewers' yeast.[3] Dried brewers' yeast and combinations of dried solubles and dried brewers' yeast were compared as supplements to corn. Although yeast (lot 3) was no better than corn (lot 1) as the sole source of protein, it effectively supplemented corn (lot 6). When combinations of yeast and dried solubles were used as supplements to corn, it was found that the supplemental value increased progressively with the amount of yeast in the ration (lots 8–13). Maximum supplemental action was obtained in the ration of lot 13 which contained equal amounts of yeast, dried solubles and corn protein. No advantage would be gained by further increasing the yeast content as twice this amount of yeast did not supplement corn any better (lot 6). These results demonstrate that it would be nutritionally valuable to increase the yeast content of dried solubles.

"Biological value" determinations

Nitrogen metabolism studies were conducted to more critically evaluate dried solubles as a sole source of protein and as a protein supplement. Biological values were determined according to the method of Mitchell and Carman ('26) for dried solubles, dried brewers' yeast, corn, casein, a combination of dried solubles and corn, and a combination of dried solubles and yeast. The combinations were used to investigate supplemental action between these sources of protein. Two male and three female rats of approximately 150 gm. weight were used in each determination. The experiment was divided into five periods of 11 days each. The first and last periods were used as standardization periods to obtain data for calculating the endogenous and metabolic nitrogen for the intervening test periods. During the standardiza-

[3] Fleischmann's Irradiated Dry Yeast obtained from Standard Brands, Inc., New York, N. Y.

tion periods, a ration containing dried whole eggs at a 4% protein level was fed, as described by Mitchell and Carman ('26) and during the test periods rations containing specific proteins at a level of 10% were fed. The first 4 days of each test period were used to adjust the rats to the new rations and for the elimination of any food other than the new test ration from the digestive tract. The urine and feces were collected during the last 7 days of each period and analyzed for total nitrogen. The composition of the rations used in the determinations and the biological values obtained are given in table 2.

It was found that biological values for dried solubles, corn, and the combination of dried solubles and corn were relatively low in comparison to the values for yeast, casein, and the combination of yeast and dried solubles. The biological value for a combination of proteins

TABLE 2

The evaluation of the proteins of distillers' dried solubles by means of "biological value" determination.

	LOW-EGG NITROGEN	DRIED SOLUBLES	DRIED SOLUBLES DRIED YEAST	DRIED YEAST	CORN	CORN DRIED SOLUBLES	CASEIN
Ration ingredients (%)							
Yellow corn					49.0	24.5	
Corn gluten meal					12.0	6.0	
Dried whole eggs	8.5						
Dried solubles		29.8	14.9			14.9	
Dried yeast			10.3	20.5			
Casein							11.7
Sucrose	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cellu-flour [1]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dextrin	54.5	32.2	35.9	39.7	1.1	16.6	48.3
Hydrogenated vegetable oil	19.0	20.0	20.9	21.8	19.9	20.0	22.0
McCollum salts no. 185	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Total protein in ration (%) from	4.0	10.0	10.0	10.0	10.0	10.0	10.0
Corn (%)					100.0	50.0	
Dried whole eggs (%)	100.0						
Dried solubles (%)		100.0	50.0			50.0	
Dried yeast (%)			50.0	100.0			
Casein (%)							100.0
Biological value [2]		53	64	61	49	50	65
Estimated biological value [3]			57			51	
Digestibility coefficient		84	82	83	93	88	100

[1] Obtained from Chicago Dietetic Supply House, Chicago, Illinois.

[2] The standard error of the *difference* between any two biological values is 3.5. Consequently the difference necessary for significance is 7 at the 5% level and 10 at the 1% level.

[3] Estimated value if no supplemental effect exists.

may be estimated, assuming the absence of supplementation, from the average of the biological values obtained when the proteins are fed separately. In the case of the combination of yeast and dried solubles, the experimental value is greater than the estimated value, which indicates that the proteins of yeast supplement the proteins of dried solubles. The estimated value and the experimental value for the combination of dried solubles and corn are similar which shows that the proteins of dried solubles do not supplement those of corn.

Digestibility coefficients, corrected for the metabolic nitrogen of the feces were also obtained from the data used in calculating the biological value of proteins, and are given in table 2. It is apparent that the proteins of corn and casein are more easily digested than are the proteins of dried solubles and dried brewers' yeast.

Amino acid deficiencies of distillers' dried solubles

The amino acid deficiencies of dried solubles at different protein levels were ascertained by supplementation with essential amino acids. The amounts of amino acids added to the rations were based upon the recommendation of Rose ('37). Since in preliminary experiments additions of half the recommended quantities of the amino acids gave essentially the same response as full amounts, the lower level was used in these studies. In the following experiments only male rats were used and the test period was 18 days (table 3).

As positive and negative controls, casein (lot 14) and dried solubles (lot 15) were fed as sole sources of amino acids. Since the supplementing of dried solubles with all the essential amino acids plus cystine (lot 16) gave no better response than when but seven amino acids were added (lot 17), this eliminated leucine, isoleucine, threonine and valine from further consideration. However, the growth responses were approximately one-half of that obtained with casein (lot 14). This difference in response may be ascribed to differences in the palatability of the rations, if it is assumed that no additional essential amino acids or other growth factors are added by casein.

Since additions of histidine, lysine, and tryptophane (lot 18) or of lysine and tryptophane (lot 19) gave responses similar to those obtained with the addition of eleven or seven amino acids (lots 16, 17), this would indicate that histidine and the other omitted amino acids are not deficient. The omission of lysine (lot 20) definitely inhibited growth, which discloses a severe deficiency of this amino acid, while the response obtained by the omission of tryptophane (lot 21) indicates a less critical deficiency of this amino acid.

Similar tests which were conducted at a 20% protein level showed that this additional amount of protein failed to correct the deficiencies of lysine and tryptophane.

To determine the adequacy of dried solubles in amino acids other than lysine and tryptophane, the protein level of the ration was lowered to 10%. Additions of various combinations of histidine, lysine, and tryptophane to dried solubles were found to be incapable of correcting all the deficiencies at this level of protein, indicating other amino acid deficiencies at this level. The growth response in all cases was limited to little more than maintenance requirements.

TABLE 3

Amino acid deficiencies of distillers' dried solubles when fed at a 15% total protein level.

LOT	14	15	16	17	18	19	20	21
Ration ingredients (%)								
Dried solubles		44.7	44.7	44.7	44.7	44.7	44.7	44.7
Casein	17.5							
Dextrin	74.5	50.3	45.5	47.8	49.5	49.7	50.0	49.6
Hydrogenated vegetable oil	5.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Amino acids[1]								
1(+) Arginine monohydrochloride			0.1	0.1				
1(−) Cystine			0.3	0.3				
1(+) Histidine monohydrochloride			0.2	0.2	0.2		0.2	0.2
dl Isoleucine			0.5					
1(+) Leucine			0.5					
1(+) Lysine monohydrochloride			0.5	0.5	0.5	0.5		0.5
dl Methionine			0.6	0.6				
dl Phenylalanine			0.7	0.7				
dl Threonine			0.6					
1(−) Tryptophane			0.1	0.1	0.1	0.1	0.1	
dl Valine			0.7					
Per cent of total protein from								
Dried solubles		100.0	100.0	100.0	100.0	100.0	100.0	100.0
Casein	100.0							
Rats — number	5	5	5	5	5	5	5	5
Average gain (gm.) — 18 days[2]	107	13	49	54	46	49	10	40
Feed efficiency	2.32	7.60	2.84	2.89	2.91	3.06	10.18	3.54

[1] All amino acids from Merck and Company, Rahway, N. J., except arginine and cystine which were laboratory preparations.

[2] The standard error of the *difference* between any two average gains is 7.4 gm. Consequently the difference necessary for significance is 15 gm. at the 5% level and 20 gm. at the 1% level

Since the proteins of dried solubles did not supplement the proteins of corn, experiments were conducted to ascertain the limiting amino acids. The rations contained 15% protein, equal amounts contributed by corn and dried solubles. The amino acid deficiencies were corrected by the addition of 0.1% l (—) tryptophane and 0.5% l (+) lysine, giving an average growth response of 172 gm. in 6 weeks which compared favorably with the response obtained with casein (lot 4). Without supplementation with these amino acids, the gain was only 71 gm. The omission of either lysine or tryptophane impaired growth appreciably. Consequently, dried solubles fails to supplement corn because of deficiencies of both lysine and tryptophane.

It was observed that the growth responses with rations containing dried solubles, even when supplemented with indispensable amino acids, were less than with casein. Since palatability might be a factor influencing the results, a series of paired feeding experiments was conducted. The duration of these experiments was 4 weeks (table 4). When compared at 15% digestible protein levels, dried solubles when supplemented

TABLE 4

Effectiveness of amino acids and combinations of proteins in correcting the amino acid deficiencies of distillers' dried solubles as measured by the controlled food consumption method.

LOT	22	23	24	25	26	27
Ration ingredients (%)						
Yellow corn				46.6	46.6	46.6
Corn gluten meal				2.5	2.5	2.5
Dried solubles	53.5		35.7	17.8	17.8	
Casein		17.5	5.8	5.8		11.7
Dried yeast					12.3	
Dextrin	41.5	74.5	52.9	22.3	15.9	33.1
Hydrogenated vegetable oil	1.4	5.0	2.6	2.0	1.9	3.1
McCollum salts no. 185	3.0	3.0	3.0	3.0	3.0	3.0
Amino acids						
l(+) Lysine monohydrochloride	0.5					
l(—) Tryptophane	0.1					
Per cent total protein from						
Corn				33.3	33.3	33.3
Dried solubles	100.0		66.7	33.4	33.4	
Casein		100.0	33.3	33.3		66.7
Dried yeast					33.3	
Rats — number	6	6	6	6	6	6
Average gain (gm.) — 4 weeks[1]	82	81	79	100	92	104
Feed efficiency	2.79	2.74	2.80	2.70	2.99	2.61

[1] The standard error of the difference between any two average gains is 6.9 gm. Consequently the difference for significance is 14 gm. at the 5% level and 19 gm. at the 1% level.

with lysine and tryptophane (lot 22) gave a growth response comparable to casein alone (lot 23) or to casein and dried solubles (lot 24). This indicates that the addition of lysine and tryptophane corrects all the deficiencies of dried solubles at this protein level. The amino acids added by casein likewise correct the deficiencies of dried solubles, permitting a response comparable to that obtained using casein as the sole source of amino acids.

In a second paired feeding experiment, it was found that a combination of dried solubles and yeast (lot 26) supplemented corn almost as effectively as dried solubles and casein (lot 25) or casein alone (lot 27). This shows that dried solubles may be converted into a good protein supplement for corn by the addition of yeast.

Chemical studies

To further elucidate the nutritive properties of dried solubles and of dried brewers' yeast as a supplement to dried solubles, studies were conducted to determine the amino acid contents as well as to attempt to measure the relative availability of certain amino acids as determined by digestion with proteolytic enzymes.

Fat free samples of dried solubles and dried brewers' yeast, ground to pass a hundred mesh sieve, were analyzed for a number of amino acids. Determinations for dicarboxylic acids, cystine, cysteine, arginine, histidine, lysine, and methionine, were made on acid hydrolyzed samples. As the amount of ammonia nitrogen parallels the amount of dicarboxylic acids present in the protein (Schmidt, '38), values for these amino acids were ascertained from amide nitrogen determinations. Cystine and cysteine were determined on the filtrate from the amide nitrogen determinations after decolorizing with Norite, using Doty's modification ('41a) of procedures developed by Shinohara ('35), Lugg ('32a, b) and Kassel and Brand ('38). Arginine, histidine, and lysine were precipitated with phospho-24-tungstic acid according to the Van Slyke et al. modification ('41a) of Cavett's procedure ('32) and the basic tungstates were decomposed with barium hydroxide. Lysine was estimated by manometric determinations of amino nitrogen and carboxyl carbon in a solution of bases. Carboxyl carbon was estimated by the method of Van Slyke et al. ('41b) as modified by MacFadyen ('42) and amino nitrogen was estimated by the method of Van Slyke ('29). Histidine was determined by the method of Macpherson ('42) and arginine by the procedure of Jorpes and Thoren ('32) as modified by Doty ('41a). In the filtrate from the basic phosphotungstates, phosphotungstic acid was removed by precipitation with barium hydroxide

and methionine was determined by an adaptation of the method of McCarthy and Sullivan ('41). Barium was removed from the solution by precipitation with sulphuric acid before development of color as a slight turbidity was apparent in the colored solutions if appreciable amounts of barium were present. Tryptophane determinations were made on separate samples using an adaptation of Doty's modification ('41b) of the procedures of Bates ('37) and Komm ('26). In addition to the usual blank determinations on the reagents, blank determinations were also made for the slight color which develops as a result of the

TABLE 5

Comparison of relative availability of certain amino acids in distillers' dried solubles and dried brewer's yeast

AMINO ACIDS	DISTILLERS' DRIED SOLUBLES [1]			DRIED BREWERS' YEAST [2]		
	Total	Liberated by enzyme digestion		Total	Liberated by enzyme digestion	
	% of protein	% of protein	% of total	% of protein	% of protein	% of total
Arginine	2.7	0.7	25.9	5.0	0.9	18.0
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0
Cystine	1.0	0.3	30.0	0.4	0.4	100.0
Dicarboxylic acids	23.3	8.3	35.6	15.6	7.9	50.6
Histidine	1.5	0.4	26.7	1.5	0.4	26.7
Lysine	3.5	1.9	54.3	6.2	3.9	62.9
Methionine	1.6	0.8	50.0	1.1	0.5	45.5
Tryptophane	0.5	0.1	20.0	0.7	0.2	28.6
Tyrosine	5.0	1.7	34.0	3.5	1.1	31.4

[1] Contained 40.35% protein (N × 6.25) on moisture and fat free basis.
[2] Contained 51.53% protein (N × 6.25) on moisture and fat free basis.

action of concentrated acid on the samples. Tyrosine was estimated after alkaline hydrolysis by a modification (Doty, '41b) of procedures described by Folin and Marenzi ('29) and Lugg ('37). The KWSZ photometer was used in all colorimetric determinations. The results of the analyses are given in table 5. The values reported are averages of triplicate determinations.

Investigations were conducted to determine the relative availability of these amino acids as measured by their liberation upon digestion with the proteolytic envmes of pancreatin.[4] An arbitrary period of 24 hours was employed for the digestion of samples and also for the subsequent dialysis. The following digestion procedure was employed.

Add 100 ml. of boiling water to each of a series of samples equivalent to 2 gm. of protein. Maintain temperature at 90°C. for 5 minutes to in-

[4] U.S.P. Pancreatin obtained from Merck and Company, Rahway, N. J.

activate enzymes and to reduce bacterial contamination. Cool and adjust to pH 7.5 with sodium hydroxide. Add 25 ml. of a potassium dihydrogen phosphate-sodium monohydrogen phosphate buffer solution adjusted to pH 7.5. Dilute to 200 ml. and add 2 ml. of pancreatin solution containing 25 mg. of pancreatin per milliliter. Add chloroform, stopper the flask, and digest 24 hours at 37°C. in a constant temperature bath. Remove flasks from the bath, adjust to pH 3.0 with hydrochloric acid and inactivate pancreatin by heating at 90°C. for 5 minutes. Centrifuge to remove insoluble material, concentrate under reduced pressure, and make to volume.

To remove undigested proteins and polypeptides, aliquots of the solutions were dialyzed for 24 hours in a continuous dialyzing apparatus developed by Westfall and Hauge ('41), utilizing a cellophane membrane.[5] The dialysates were concentrated under reduced pressure, made to volume, and analyzed for total nitrogen, using an adaptation of the micro-Kjeldahl method of Ma and Zuzaga ('42). Since successive samples gave dialysates with the same total nitrogen content, composite samples were prepared and concentrated under reduced pressure so that the increased concentration of amino acids would be suitable for analysis. Aliquots were hydrolyzed with HCl (constant boiling mixture) for 6 hours to free any amino acids still present in the form of peptides. Amino acid analyses were made by the methods previously described.

The results of the chemical determination show that yeast protein contains considerably more of the particular amino acids which were found to be deficient in dried solubles protein by biological assays, namely, lysine and triptophane (table 5). The proteins of dried solubles contain only 56% as much lysine and 71% as much tryptophane as is found in yeast protein. Furthermore, the digestion experiments with pancreatin showed that these amino acids were liberated from the proteins of dried solubles to a lesser extent than from yeast. Since it is impossible to simulate the conditions in the digestive tract by in vitro experiments, these values should be considered only as relative indices of the availability of the amino acids. However, as the total amounts of lysine and tryptophane of dried solubles protein as well as the respective percentages liberated were less than that of yeast protein, this offers an explanation of the nutritional limitations of dried solubles, as compared to yeast.

[5] Cellophane dialyzing tubing (19 mm. dia.) obtained from E. H. Sargent and Co., Chicago, Ill.

DISCUSSION

It is recognized that there may be some variation in both the quantity and quality of the proteins in this type of product as prepared by different companies and even by the same company. These variations may be due to the type and proportion of cereal grains constituting the mash, the composition of these grains, the proportionate content of yeast in the final product, and the recovery procedure used.

The possibility of using dried solubles as a protein supplement in the ration of farm animals is limited by its deficiency in certain essential amino acids. By means of biological assays it was ascertained that lysine and tryptophane were the limiting amino acids for growth when this product was fed at a 15% level. The requirements of the animal for these amino acids could not be satisfied by increasing the protein level to 20%. When the protein content was lowered to a 10% level, other amino acid deficiencies became apparent. In studying the supplementing value of dried solubles for corn, it was found that the dried solubles product does not supplement corn because of deficiencies of lysine and tryptophane. Although the lysine and tryptophane content of yeast is higher than that of the cereal grains, the amount of yeast in dried solubles is not adequate to contribute enough of these amino acids to sufficiently enhance the protein quality of this product to make it valuable either as a sole source of protein or as a supplement to corn.

Since Joseph et al. ('43) have previously demonstrated that the protein content of corn stillage could be increased by increasing the yeast content by fermentation methods, a study was made of the nutritional value of dried brewers' yeast and of various combinations of yeast and dried solubles for the purpose of determining the possible role of yeast in the fortification of dried solubles. It was found that yeast was of greater nutritional value than dried solubles when used as a sole source of protein and effectively supplemented dried solubles, corn and combinations of these products. Chemical studies also reveal that yeast is superior to dried solubles. The total amounts of lysine and tryptophane as well as the relative amounts of these amino acids liberated by proteolytic enzymes were found to be greater in yeast than in dried solubles. Since it was found that additions of small amounts of yeast to dried solubles definitely increased growth, productions of dried solubles with a higher content of yeast would be desirable from a nutritional viewpoint.

Although the dried solubles product used in these experiments was decidedly deficient in lysine and tryptophane, it is possible that it could be used as a source of protein in rations of farm animals providing these deficiencies are corrected by other constituents of the ration.

SUMMARY

The nutritive value of distillers' dried solubles as a source of protein was evaluated by means of biological tests with rats and by chemical analyses. It was found to be inadequate as the sole source of protein and as a protein supplement because of deficiencies of lysine and tryptophane.

Since the proteins of solubles are derived from the protein of yeast and cereal grains, a study was made of the nutritive value of dried brewers' yeast and various combinations of yeast and solubles for the purpose of determining the value of yeast in the fortification of solubles. It was found that yeast was of greater nutritive value than dried solubles as the sole source of protein and effectively supplemented solubles, corn and combinations of these products.

Chemical studies revealed that solubles contain considerably less lysine and tryptophane than yeast. Furthermore, digestion experiments with proteolytic enzymes indicated that these amino acids are less available in solubles than in yeast. Since it was found that the addition of small amounts of yeast to dried solubles definitely increased its nutritive value, production of dried solubles with a higher yeast content would be desirable.

LITERATURE CITED

BATES, R. W. 1937 A rapid method for the quantitative determination of tryptophane. J. Biol. Chem., Proceedings, vol. 119, p. vii.

BAUERNFEIND, J. C., J. C. GAREY, W. BAUMGARTEN, L. STONE AND C. S. BORUFF 1944 Nutrient content of alcohol fermentation by-products from corn. Ind. Eng. Chem., vol. 36, p. 76.

BORUFF, C. S., A. F. LANGLYKKE AND S. BLACK 1940 Vitamin content of distiller's by-products. Ind. Eng. Chem., vol. 32, p 1237.

CARTER, H. E., AND G. E. PHILLIPS 1944 Nutritive value of yeast proteins. Federation Proceedings, vol. 3, p. 123.

CAVETT, J. W. 1932 A modification of the Van Slyke nitrogen distribution method. J. Biol. Chem., vol. 95, p. 335.

DOTY, D. M. 1941a Methods for estimation of some amino acids in corn grain. Ind. Eng. Chem. Anal. Ed., vol. 13, p. 169.

———— 1941b The tyrosine, tryptophane, cystine, arginine and histidine content of single cross hybrid strains of corn. Ph. D. thesis: Purdue Univ., pp. 29–30.

FOLIN, O., AND A. D. MARENZI 1929 Tyrosine and tryptophane determinations on one-tenth gram of protein. J. Biol. Chem., vol. 83, p. 89.

JORPES, E., AND S. THOREN 1932 The use of the Sakaguchi reaction for the quantitative determination of arginine. Biochem. J., vol. 26, p. 1504.

JOSEPH, N. R., P. J. KOLACHOV AND R. T. WILLKIE 1942 Proteins of grain distillery by-products. Div. of Biol. Chem., Am. Chem. Soc. Meeting, Buffalo.

JOSEPH, N. R., A. NOVAK AND J. K. WOODS 1943 Production of a food concentrate from distillery residues. Div. of Agr. and Food Chem., Am. Chem. Soc. Meeting, Pittsburgh.

KASSELL, B., AND E. BRAND 1938 The photometric determination of cystine, cysteine; ascorbic acid and related compounds with phosphotungstic acid. J. Biol. Chem., vol. 125, p. 115.

KOMM, E. 1926 The tryptophan-aldehyde reaction. V. Method for the determination of tryptophan and the tryptophan content of some proteins. Z. Physiol. Chem., vol. 156, p. 202.

LUGG, J. W. 1932a The application of phospho-18-tungstic acid (Folin's reagent) to the colorimetric determination of cysteine, cystine and related substances. I. The reduction of phospho-18-tungstic acid by various substances. Biochem. J., vol. 26, p. 2144.

———— 1932b II. The determination of sulphydryl compounds and disulfides already existing in solution. Biochem. J., vol. 26, p. 2160.

———— 1937 On the use of mercuric salts and nitrous acid in the colorimetric determination of tyrosine and tryptophan present in solution. Biochem. J., vol. 31, p. 1422.

MACFADYEN, D. A. 1942 Determination of amino acids in plasma by the ninhydrin-carbon dioxide reaction without removal of proteins. J. Biol. Chem., vol. 145, p. 387.

MACPHERSON, H. T. 1942 Modified procedure for the colorimetric estimation of arginine and histidine. Biochem. J., vol. 36, p. 59.

McCARTHY, T. E., AND M. X. SULLIVAN 1941 A new and highly specific colorimetric test for methionine. J. Biol. Chem., vol. 141, p. 871.

MA, T. S., AND G. ZUAZAGA 1942 Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. Ind. Eng. Chem. Anal. Ed., vol. 14, p. 280.

MITCHELL, H. H., AND G. G. CARMAN 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., vol. 68, p. 183.

ROSE, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, vol. 86, p. 298.

SCHMIDT, C. L. A. 1938 The Chemisty of Amino Acids and Proteins. P. 204. Charles C. Thomas, Springfield, Illinois.

SHINOHARA, K. 1935 The determination of thiol and disulfide compounds, with special reference to cysteine and cystine. II. A critical study of the relation between phospho-18-tungstic acid reagent and other substances than thiol compounds. J. Biol. Chem., vol. 110, p. 263.

SLOAN, H. J. 1941 The use of distillers' by-product in poultry rations. Poultry Sci., vol. 20, p. 83.

SYNOLD, R. E., C. W. CARRICK, R. E. ROBERTS AND S. M. HAUGE 1943 Distillers' dried solubles as a vitamin supplement in chick rations. Poultry Sci., vol. 22, p. 323.

VAN SLYKE, D. D. 1929 Manometric determination of primary amino nitrogen and its application to blood analysis. J. Biol. Chem., vol. 83, p. 425.

VAN SLYKE, D. D., A. HILLER AND D. A. MACFADYEN 1941a The determination of hydroxylysine in proteins. J. Biol. Chem., vol. 141, p. 681.

VAN SLYKE, D. D., R. T. DILLON, D. A. MACFAYDEN AND P. HAMILTON 1941b Gasometric determination of carboxyl groups in free amino acids. J. Biol. Chem., vol. 141, p. 627.

WESTFALL, R. J., AND S. M. HAUGE 1941 Unpublished data.

CALCIUM METABOLISM OF PRESCHOOL CHILDREN

ELLEN KEMP WATSON, ELINOR WHELAN McGUIRE, FRIEDA L. MEYER AND MILICENT L. HATHAWAY

New York State College of Home Economics, Cornell University, Ithaca

(Received for publication May 28, 1945)

Because of the high requirement for calcium for optimal skeletal development, and the relatively small amounts in all foods except milk and milk products, it is important to include in the diet foods which can be shown to favor calcium retention. Reports in the literature on the effect of orange juice on calcium retention are conflicting (Chaney and Blunt, '25; Daniels and Everson, '37; and Shepherd, Macy, Hunscher and Hummel, '40), making it seem important to study further the possible influence of this food, and certain constituents of it, on mineral retention. The data presented here are part of a larger study on the influence of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of eight preschool children. The effect of these supplements on ascorbic acid has been reported by Meyer and Hathaway ('44), and on citrate metabolism by Metcalf and Hathaway ('45); their effect on phosphorus and nitrogen metabolism will be reported later.

EXPERIMENTAL

Plan of the experiment. Two groups of four children each lived at the college laboratory apartment for 5 months. Subjects A, C, F, G, and H were girls, aged 55, 49, 49, 40 and 38 months, respectively, at the beginning of the study. Their respective weights at that time were 50, 38, 40, 32, and 29 pounds. Subjects B, D, and E were boys, aged 51, 44, and 55 months, and weighing 36, 35, and 34 pounds, respectively

The general plan of the complete experiment has been reported in detail by Meyer ('43), but the following facts are particularly pertinent to this part of the study. The children were maintained on a basal diet adequate in all nutrients except ascorbic acid. One teaspoonful of cod liver oil was given daily to each child. The first group of children (A, B, C, and D), were given 800 ml. of milk daily; the other group (E, F, G, and H), received only 500 ml., since it was considered that at the lower calcium level possible effects of the dietary supplements might be

more marked. Through appropriate changes in the basal diet, particularly an increase in the meat and eggs, similar levels of protein were maintained in the diets of the two groups. The calcium-phosphorus ratio was reduced slightly, from 0.9 to 0.8, on the lower intake of milk. The first 4 weeks were considered a period of personal adjustment to the new surroundings, and of metabolic adjustment to the diet. During these preliminary periods the children each received at least 1 gm. of calcium daily, the low-milk group being given an additional 0.268 gm. of calcium per day as calcium gluconate (two 1.5 gm. tablets [1] per day), in order to assure comparable calcium stores in all eight children. Somewhat smaller amounts of the basal foods were consumed by subject H throughout the study, and by subject G during the first 4 and last 5 weeks, but since milk and all supplements were given at the same level to all subjects in a group, there was little difference in the calcium intake for the different members of a group. Slight increases in the calcium content of the milk as analyzed account for the somewhat higher calcium intakes during periods 9 and 19 for the high-milk group, and 12 and 17 for the low-milk group. Supplements of crystalline ascorbic acid (100 mg.), potassium citrate (3.38 gm.) or both were given as indicated in table 1. These amounts correspond roughly to the amounts of ascorbic acid and of citrate-ion found in 200 ml. of orange juice. During the last 2 weeks the low-milk group was given orange juice in place of the crystalline supplements.

Collection and preparation of samples for analyses. The collection and preparation of urine and food samples have been described by Metcalf and Hathaway ('45). Feces were marked with carmine. The combined feces for each weekly period were digested with 1:4 hydrochloric acid according to the method of Stearns ('28–'29), made to 2000 ml. and aliquots stored for analysis.

Calcium determinations. The calcium content of the foods and excreta was determined using the following modifications of the McCrudden ('11) method. The ash from the foods and feces samples was fused with sodium carbonate according to the method of Frear and Kahlenberg ('33). To remove the supernatant from the precipitated calcium oxalate, Pyrex sintered-glass immersion tubes of fine porosity were used the second year. Hot normal sulphuric acid was then used to dissolve the calcium oxalate, and to assure its complete removal from the immersion tube.

[1] Squibb

RESULTS AND DISCUSSION

The data from this study are presented in table 1. Data for the preliminary adjustment periods have been omitted. The calcium retentions during these four periods were generally higher than those during the periods reported, but were not consistently so.

Calcium retentions. Before considering whether the various supplements were important in determining the calcium retained by these children, it seemed advisable to ascertain that the calcium retentions were normal. Average retentions of 112 ± 36 mg. to 217 ± 154 mg. have been reported for children of this age range (Macy, '42; Outhouse et al., '39; Kinsman et al., '39; Pierce et al., '40; Hawks et al., '42; and Daniels, '41). The average retentions for the eight children over the 16 weeks of the present study ranged from 76 to 156 mg. per day. The values were 131 ± 50 mg. for the four children on the high-milk intake, and 96 ± 26 mg. for those on the low-milk intake. In each group one child had values much lower than the rest, accounting for the rather large standard deviation in the values. Although these values are below some of the average values they are well within the ranges reported and it may be assumed that the calcium metabolism of the eight subjects was normal.

A summary of the calcium balances in mg./kg. body weight is given in table 2. When the data are grouped according to intake per kilogram (subjects A, E, F, and G, 39 to 50 mg./kg.; subject H, 52 to 56 mg./kg.; and subjects B, C, and D, 59 to 68 mg./kg.), the retentions are somewhat more related to the intakes: 3.2 to 6.4 mg. at the lowest level, 6.5 to 10 mg. for subject H, and 5.6 to 11 mg. for the highest level. Although the intakes for subject H are intermediate, the retentions correspond to those found for the children on the highest intake. Compared with the values for the mean daily calcium retentions reported in other studies (Macy, '42; Outhouse et al., '39) the mg./kg. values for all but subject A seem to be normal. She was overweight when she came on the study, and her retentions per kilogram are naturally lower than if her weight had been normal.

Effect of ascorbic acid supplement on calcium retention. In a review, Reid ('43) has summarized the interrelationships of calcium and ascorbic acid in various metabolic processes (growth, water balance, cell permeability, and muscle action) and has stated that a deficiency of either of these substances may affect adversely the retention or metabolism of the other. Any effect of an excess of ascorbic acid on the retention of calcium should be apparent in a comparison of the results obtained with diets I and II, and diets III and IV, table 1. The results

TABLE 1

Average daily calcium balance for four preschool children on high-milk intake.

SUBJECT		A				B				C				D			
Diet	Period	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention	Intake	Excretion Urine	Excretion Feces	Retention
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
I[1]	5	1128			. .	1127	139	870	118	1128	132	874	122	1125	110	919	97
	6	1128	36	1037	55	1124	141	814	169	1127	142	890	95	1125	120	941	64
	10	1081	21	1003	57	1079	122	833	124	1081	146	881	54	1076	89	899	88
	11	1108	24	983	101	1102	130	863	109	1108	149	878	81	1101	109	786	206
	12	1145	27	993	124	1141	139	848	153	1145	149	841	154	1141	134	840	167
	Av.	1118	27	1004	84	1115	134	846	135	1118	144	873	101	1114	112	877	124
II[2]	7	1081	34	1000	48	1078	135	868	75	1081	144	809	128	1076	111	799	167
	8	1122	36	1057	28	1120	138	823	159	1121		.	.	1117	120	841	156
	9	1186	24	1050	113	1185	135	870	180	1187	150	862	175	1182	115	807	260
	13	1118	30	1019	68	1113	160	862	91	1116	142	858	116	1114	147	822	145
	14	1121	31	987	103	1114	153	841	121	1118	152	835	131	1114			.
	Av.	1126	31	1023	72	1122	144	853	125	1125	147	841	137	1121	123	817	182
III[3]	15	1105	17	1020	68	1102	100	883	119	1104	104	817	183	1103	82	858	164
	16	1127	14	967	146	1122	92	882	148	1125	104	817	203	1122	76	791	255
	17	1123	12	1020	92	1119	89	864	165	1122	97	937	88	1119	82	950	88
	Av.	1118	14	1002	102	1114	94	876	144	1117	102	857	158	1115	80	866	169
IV[4]	18	1096	8	973	116	1092	92	855	145	1095	95	786	214	1092	72	811	209
	19	1164	13	990	160	1160	103	921	137	1162	108	866	189	1160	87	911	162
	20	1137	9	1040	89	1133	77	936	120	1136	85	838	213	1135	84	935	115
	Av.	1132	10	1001	122	1128	91	904	134	1131	96	830	205	1129	81	886	162
Gen. av.		1123	22	1009	91	1119	122	865	133	1122	127	853	143	1119	103	861	156

Average daily calcium balance for four preschool children on low-milk intake.

SUBJECT		E				F				G				H			
I[1]	7	796	47	642	107	796	76	611	109	796	28	665	103	759	30	539	190
	8	808	46	662	100	776	76	611	89	776	26	682	68	773	30	626	117
	9	800	46	709	45	765	77	628	60	765	28	658	79	766	29	623	114
	Av.	801	46	671	84	779	76	617	86	779	27	668	83	766	30	596	140
II[2]	5	776	67	625	84	778	85	588	105	778	36	673	69	744	36	575	133
	6	762	52	670	40	765	81	593	91	766	34	654	78	733	36	604	93
	10	819	53	696	70	787	78	608	101	786	29	661	96	787	33	634	120
	11	811	46	672	93	779	71	606	102	766	21	633	112	768	22	633	113
	12	834	52	701	81	802	73	650	79	799	25	674	100	800	27	631	142
	Av.	800	54	673	74	782	78	609	96	779	29	659	91	766	31	615	120
III[3]	13	773	36	664	73	741	55	559	127	735	15	611	109	742	17	627	98
	14	779		.	.	748	60	574	114	741	18	615	108	745	13	646	86
	15	794	32	667	95	759	64	615	80	749	19	628	102	752	12	627	113
	Av.	782	34	665	84	749	60	583	107	742	17	618	106	746	14	633	99
IV[4]	16	811	37	716	58	778	70	664	44	736	16	643	77	760	14	669	77
	17	850	34	727	89	818	58	615	145	775	17	634	124	798	14	678	106
	18	792	26	717	49	753	53	593	107	714	19	589	106	739	17	624	98
	Av.	818	32	720	65	783	60	624	99	742	17	622	102	766	15	657	94
V[5]	19	806	46	685	75	771	71	627	73	730	25	587	118	741	22	623	96
	20	825	49	693	83	791	82	574	135	738	23	657	58	756	22	621	113
	Av.	815	47	689	79	781	76	600	104	734	24	622	88	748	22	622	104
Gen. av.		804	45	683	76	775	71	607	98	759	24	642	94	760	23	624	113

[1] Basal diet only.
[2] Basal diet plus ascorbic acid.
[4] Basal diet plus ascorbic acid and potassium citrate.
[5] Basal diet plus orange juice.

TABLE 2

Summary of calcium balances for eight preschool children.

		INTAKE	EXCRETION Urine	EXCRETION Feces	RETENTION	% of intake
		mg./kg.	mg./kg.	mg./kg.	mg./kg.	
Subject A						
Diet	I [1]	49	1.2	44	3.7	8
	II [2]	50	1.4	45	3.2	6
	III [3]	49	0.6	44	4.5	9
	IV [4]	49	0.4	44	5.3	11
Subject B						
Diet	I [1]	67	8.1	51	8.1	12
	II [2]	68	8.7	51	7.5	11
	III [3]	65	5.5	51	8.4	13
	IV [4]	65	5.3	52	7.7	12
Subject C						
Diet	I [1]	62	8.0	49	5.6	9
	II [2]	62	8.1	46	7.5	12
	III [3]	59	5.4	45	8.4	14
	IV [4]	60	5.1	44	10.8	18
Subject D						
Diet	I [1]	67	6.7	53	7.4	11
	II [2]	66	7.2	48	10.7	16
	III [3]	63	4.5	49	9.5	15
	IV [4]	63	4.5	49	9.0	14
Subject E						
Diet	I [1]	50	2.9	42	5.2	10
	II [2]	49	3.3	42	4.6	9
	III [3]	47	2.0	40	5.1	11
	IV [4]	48	1.9	42	3.8	8
	V [5]	47	2.7	40	4.6	10
Subject F						
Diet	I [1]	42	4.1	33	4.6	11
	II [2]	42	4.2	33	5.1	12
	III [3]	39	3.1	31	5.6	14
	IV [4]	40	3.1	32	5.1	13
	V [5]	39	3.8	30	5.3	13
Subject G						
Diet	I [1]	49	1.7	42	5.3	11
	II [2]	49	1.8	41	5.7	12
	III [3]	45	1.0	37	6.4	14
	IV [4]	45	1.0	38	6.2	14
	V [5]	44	1.5	38	5.3	12
Subject H						
Diet	I [1]	56	2.2	43	10.1	18
	II [2]	56	2.2	45	8.7	16
	III [3]	53	1.0	45	7.0	13
	IV [4]	53	1.1	46	6.5	12
	V [5]	52	1.5	43	7.2	14

[1] Basal diet only.
[2] Basal diet plus ascorbic acid.
[3] Basal diet plus potassium citrate.
[4] Basal diet plus ascorbic acid and potassium citrate.
[5] Basal diet plus orange juice.

were inconsistent: the calcium retention was increased for subjects D, F, and G when ascorbic acid was added to the basal diet, but not when it was added to the basal diet plus potassium citrate, but the reverse was true for subject A. The retentions for subjects B, E, and H were less when ascorbic acid was added to both of the diets, but were more for subject C in both cases. Comparison of retention values for all the periods when ascorbic acid was added (diets II and IV) with those when it was omitted (diets I and III) showed that the differences were not statistically significant for any child. These results check those of Daniels and Everson ('37) rather than those of Shepherd and associates ('40), i.e., ascorbic acid in excess of that in the basal diet did not significantly increase the retention of calcium in these preschool children.

Effect of potassium citrate supplement on calcium retention. Studies by Shohl ('37) and Hamilton and Dewar ('37) have shown that citric acid and its salts have antirachitogenic action in the rat. The work of Hathaway and Meyer ('39) confirmed the effectiveness of citrates in improving calcification and indicated that potassium citrate was more effective than the sodium salt or a mixture of citric acid and its sodium salt. It seemed possible, therefore, that the citrates present in orange juice might have been a factor in the increased calcium retention found in some human subjects when orange juice was added to the diet. Consequently 3.38 gm. of potassium citrate were added to the diets of these 8 children. This is the amount calculated to contain the same citrate-ion content as 200 mg. of orange juice. The results are given under diets III and IV in table 1.

In every period during which potassium citrate was given (diets III and IV) the urinary calcium excretion was less than when it was omitted. The fecal excretions during these periods, and consequently the retentions of calcium, were irregular. The retentions for subject C were significantly higher when the salt was added, those for subject H were significantly higher without the salt, and the differences were not statistically significant for the other six children. It may be concluded that although potassium citrate ingestion significantly reduces the urinary excretion of calcium, undetermined factors affecting its absorption from the intestine may overbalance this decreased urinary excretion so that the retention may be little affected.

Effect of orange juice supplement on calcium retention. An amount of orange juice containing 110 mg. of ascorbic acid (averaging 218 ml.) was added to the basal diet of one group of the children during the last two periods, and the results are recorded under diet V, part 2 of table 1. The average retentions of three of the four children were

slightly higher during these two periods than in the preceding three periods on potassium citrate plus ascorbic acid, but not significantly so. The urinary excretions were higher in all cases than on the potassium citrate supplement, but the fecal excretions were sufficiently lower to result in slightly higher retentions. The differences in response for the periods on orange juice supplement make questionable the significance of these increases in retention.

Again these results agree with those of Daniels and Everson ('39) rather than with those of Shepherd and associates ('40). In the studies of Chaney and Blunt ('25) and those of Ludwig and Schuck ('41) on older subjects, only short preliminary periods and short experimental periods were used, so that they are not comparable with the present study.

Fecal excretion and intestinal absorption of calcium. The average daily fecal excretions of calcium ranged from 853 to 1009 mg. on the high-milk intake, and from 607 to 683 on the low-milk intake. Greater variation was found from period to period, and from child to child than was found for the urinary excretions of calcium. This was due, in part at least, to the difficulty in separation of feces between periods, and to the much higher concentration of calcium in fecal material.

Marked differences in rate of defecation and nature of the stools was noted among the eight children: subjects D and F were frequently constipated, and subjects E and H often had unformed stools. Fecal calcium excretion, however, did not show much relationship to rate of defecation: e.g., subject A with normal defecation, excreted the most calcium in the feces, and subject H, with rapid defecation averaged less fecal calcium than subject G.

Since there is extensive evidence that calcium is not actively excreted into the intestinal tract, the intake minus the fecal output may be considered to be the calcium absorbed. On this basis, the average daily absorptions were 113, 255, 269 and 258 mg. or 10 to 24% of the intake for the high-milk group, and 112, 169, 118 and 136 mg. or 14 to 22% of the intake for the low-milk group. The absorption for A was abnormally low in comparison with that of the other three subjects in her group, and her retention was of necessity much below theirs. Subjects C and F, however, who apparently absorbed more calcium than the other subjects in their groups, did not have higher retention values. Since the urinary output of subjects A, G, and H averaged only 22 to 24 mg. per day, it may be that absorption was one factor affecting the amount which they retained. For the other five subjects, however, this would not appear to be the main controlling mechanism.

Urinary excretion of calcium. The urinary excretions of calcium were not uniform from child to child, but were relatively consistent for each child on a given supplement. They were little affected by additions of ascorbic acid to either the basal diet or to the basal diet plus potassium citrate, but the addition of potassium citrate (diets III and IV) resulted in significantly lower urinary calcium excretions in all eight children. This decrease averaged from 10 to 53 mg. for the different subjects, but was relatively consistent and characteristic for each child. These results lend support to the suggestion that urinary calcium excretion may be related to acid-base balance. Addition of potassium citrate to the diet increased the average urinary pH by 0.9 to 1.4 units, from averages of 5.5 to 5.9, to from 6.7 to 6.9. With an increased urinary pH, the urinary calcium excretion was reduced. When orange juice was added to the diet of the low-milk group, the urinary excretion of calcium was about the same as on the basal diet for E and F, but somewhat lower for G and H. It was higher than on the potassium citrate supplement in all subjects. The addition of orange juice increased the basal pH values by about 0.3 units for E and F, and by about 0.6 units for G and H. These results also lend possible support to a relationship between urinary calcium excretion and acid-base balance.

Knapp and Stearns ('44) state that urinary calcium bears an important relationship to calcium intake and body weight. According to their formulas for calculation of the normal percentage of calcium intake which is excreted in the urine, the children from the high-milk group should have values between 1.0 and 16%, and those from the low-milk group between 1.2 and 19%. The percentage values found were as follows: A, 0.8 to 3.2; B, 6.8 to 14; C, 7.7 to 14; D, 6.6 to 13; E, 3.2 to 8.6; F, 7.4 to 11; G, 2.2 to 4.6; and H, 1.6 to 4.9. Thus subjects A, G, and H eliminated amounts of calcium definitely in the lower normal range; subjects B, C, and D definitely in the higher normal range; and only subjects E and F in the middle range, regardless of supplement. Knapp and Stearns recognized that the major factor regulating urinary excretion is endogenous, and believe that it is probably related to endocrine balance. Parathyroid and thyroid activity may be involved, but the exact relationship has not been determined.

SUMMARY AND CONCLUSIONS

Calcium metabolism has been studied in eight preschool children, four on average calcium intake of 1122 mg., and four on an intake of 775 mg. The basal diet was supplemented with ascorbic acid, potassium citrate, and orange juice as indicated. The results were as follows:

1. Calcium retentions during 16 weeks averaged 131 $\pm$ 50 mg. on the higher level of intake, and 96 $\pm$ 26 mg. on the lower level.

2. The addition of 100 mg. of ascorbic acid to a diet containing 23 to 25 mg. of the vitamin did not significantly increase the retention of calcium.

3. The addition of 3.38 gm. of potassium citrate lowered the urinary excretion of calcium in all eight subjects but did not significantly alter the calcium retention.

4. On the substitution of orange juice for crystalline ascorbic acid and potassium citrate supplements, the retentions of calcium were not significantly altered.

5. In at least five of the eight subjects the absorption of calcium did not appear to be the main factor regulating its retention.

6. In these eight children factors other than diet alone regulated calcium retention during the 16 weeks of observation.

LITERATURE CITED

CHANEY, M. S., AND K. BLUNT 1925 The effect of orange juice on the calcium, phosphorus, magnesium and nitrogen retention and urinary organic acids of growing children. J. Biol. Chem., vol. 66, p. 829.

DANIELS, A. L. 1941 Relation of calcium, phosphorus and nitrogen retention to growth and osseous development. A long time study of three preschool boys. Am. J. Dis. Children, vol. 62, p. 279.

DANIELS, A. L., AND G. J. EVERSON 1937 The relation of ascorbic acid ingestion to mineral metabolism in children. J. Nutrition, vol. 14, p. 317.

FREAR, D. E. H., AND O. J. KAHLENBERG 1933 A study of the accuracy of the McCrudden method for calcium and magnesium in biological materials. J. Biol. Chem., vol. 100, p. 85.

HAMILTON, B., AND M. M. DEWAR 1937 Effect of citrate and tartrate on experimental rickets. Am. J. Dis. Children, vol. 54, p. 548.

HATHAWAY, M. L., AND F. L. MEYER 1939 Citric acid and its salts as calcifying agents in rats. J. Nutrition, vol. 17, p. 419.

HAWKS, J. E., M. M. BRAY, M. O. WILDE AND M. DYE 1942 The interrelationship of calcium, phosphorus and nitrogen in the metabolism of preschool children. J. Nutrition, vol. 24, p. 283.

KINSMAN, G., D. SHELDON, E. JENSEN, M. BERNDS, J. OUTHOUSE AND H. H. MITCHELL 1939 The utilization of the calcium of milk by preschool children. J. Nutrition, vol. 17, p. 429.

KNAPP, E. L., AND G. STEARNS 1944 Studies of urinary calcium excretion. I. In normal persons. Fed. Proc., vol. 3, p. 59.

LUDWIG, C. E., AND C. SCHUCK 1941 The influence of orange juice on calcium retention in adults. J. Home Economics, vol. 33, p. 600.

McCRUDDEN, F. H. 1911 The determination of calcium in the presence of magnesium and phosphates: the determination of calcium in urine. J. Biol. Chem., vol. 10, p. 187.

MACY, I. G. 1942 Nutrition and chemical growth in childhood, vol. 1. Evaluation. Charles C. Thomas, Springfield, Ill.

METCALF, E. R., AND M. L. HATHAWAY 1945 Citrate metabolism in preschool children. J. Nutrition, vol. 29, p. 211.

MEYER, F. L. 1943 Ascorbic acid requirement and metabolism of preschool children. Thesis, Cornell University.

MEYER, F. L., AND M. L. HATHAWAY 1944 Further studies on the vitamin C metabolism of preschool children. J. Nutrition, vol. 28, p. 93.

OUTHOUSE, J., G. KINSMAN, D. SHELDON, I. TWOMEY, J. SMITH AND H. H. MITCHELL 1939 The calcium requirements of five preschool girls. J. Nutrition, vol. 17, p. 199.

PIERCE, H. B., R. G. DAGGS, A. B. MESERVEY AND W. J. SIMCOX 1940 The retention of calcium and phosphorus by preschool children. J. Nutrition, vol. 19, p. 401.

REID, M. E. 1943 Interrelations of calcium and ascorbic acid to cell surfaces and intercellular substances and to physiological action. Physiol. Rev., vol. 23, p. 76.

SHEPHERD, M. L., I. G. MACY, H. A. HUNSCHER AND F. C. HUMMEL 1940 Synthesized, processed and natural sources of vitamin C in the mineral metabolism of normal children. J. Ped., vol. 16, p. 704.

SHOHL, A. T. 1937 The effect of the acid-base content of the diet upon the production and cure of rickets with special reference to citrates. J. Nutrition, vol. 14, p. 69.

STEARNS, G. 1928–1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analyses. J. Lab. and Clin. Med., vol. 14, p. 954.

THE PROTEIN REQUIREMENTS OF ADULT HUMAN SUBJECTS IN TERMS OF THE PROTEIN CONTAINED IN INDIVIDUAL FOODS AND FOOD COMBINATIONS [1]

MILDRED BRICKER,[2] H. H. MITCHELL AND GLADYS M. KINSMAN
Division of Animal Nutrition and the Department of Home Economics, University of Illinois, Urbana

ONE FIGURE

(Received for publication May 31, 1945)

Most of what we know, or think we know, about human nutrition has been obtained from experimental observations upon laboratory animals. Nutrition experiments upon human subjects are difficult to carry out, because of the inconvenience to the subjects of the conditions that must be imposed for the most effective interpretation of the results to be secured, not the least of which is the great divergence between the experimental diets that must be used and the diets to which the subjects have been habituated. This is particularly true of studies in protein nutrition in which low-protein diets must be tolerated for many days. The usual diets consumed by adults contain two or three (or more) times the amount of protein required for nitrogen equilibrium.

Determinations of protein utilization in digestion and in metabolism upon growing rats are readily made and the measurements obtained may be accurate from the standpoint of reproducibility. However, there is always some degree of doubt as to their applicability to the human, and until more information on the value of the rat as a "pilot" animal in human nutrition is at hand, this doubt will remain. Meantime there is a real need for protein studies on human subjects.

A number of experimental methods have been employed in studies of protein nutrition in adult men and women: the method of Thomas ('09) involving the computation of biological values in the original meaning of the term; the method of Murlin and associates (Sumner, Pierce and

[1] The authors gratefully acknowledge the assistance in carrying out this experiment of funds donated by the Graduate School of the University of Illinois.

[2] The data reported in this paper were taken from a thesis presented by Mildred Bricker to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy.

Murlin, '38) in which the Thomas method is modified by use of an egg protein diet as a standard rather than a nitrogen-free diet; and the method of Sherman (Sherman, Winters and Phillips, '19), in which different sources of protein are compared on the basis of the balances of nitrogen secured on comparable, and low, intakes of nitrogen. None of these methods is ideal in experimentation with adult human subjects, either because of the difficulty in tolerating the experimental diets, the purely comparative nature of the results secured, or the impossibility of computing a protein requirement for nitrogen equilibrium.

However, if the relationship in adult nutrition between the nitrogen intake and the nitrogen balance (or ouput) is rectilinear below the point of equilibrium, and even somewhat above it, a more practicable method is at hand for studying with human subjects the utilization of dietary nitrogen and the requirements for equilibrium of nitrogen from different dietary sources. Martin and Robison ('22) established the rectilinear character of the relationship for human adults between the nitrogen intake and output when the dietary nitrogen was supplied by whole wheat flour, and less certainly when it was furnished by milk. In their selection from the literature of metabolism data for the determination of the protein requirement for maintenance in man, Leitch and Duckworth ('37) were able to describe satisfactorily the relationship between nitrogen intake and nitrogen output by straight lines. Melnick and Cowgill ('37) report remarkably close rectilinear relationships for adult dogs between nitrogen intake and nitrogen balance for dietary proteins differing widely in biological value, i.e., lactalbumin, serum protein, casein and gliadin. The intercept of the straight lines describing this relationship on the abscissa representing nitrogen equilibrium, gives the amount of nitrogen, of the species present in the diet, required for equilibrium. The intercept on the ordinate erected at zero nitrogen intake, gives the minimum endogenous output of nitrogen in the urine plus the metabolic nitrogen in the feces. This method was used by Harris and Mitchell ('41) in estimating the requirements of nitrogen in the form of urea and of casein for the maintenance of nitrogen equilibrium in adult sheep. It was used in the investigation on human subjects to be reported in this paper.

EXPERIMENTAL PROCEDURES

The subjects of the experiment were nine young women attending the University of Illinois. Their description with reference to age, weight, height and basal metabolism is given in table 1. The basal metabolism was determined by the gasometer method. Two or more

measurements were made upon each subject, depending upon the agreement secured between the first two. The nitrogen metabolism data for different subjects were pooled together by expressing them in milligrams per basal calorie in accordance with the observations of Smuts ('35) confirming those of Terroine that the minimum endogenous catabolism of nitrogen, to which the maintenance requirement of protein is due, varies with the basal metabolism.

The experimental diets were planned to contain as high a proportion of the total nitrogen in the test food as possible. The proportion realized was generally 95% or higher, though in a few cases it ranged from 91

TABLE 1

Description of the subjects of the experiments.

SUBJECTS		AGE	WEIGHT	HEIGHT	SURFACE AREA [1]	BASAL METABOLISM	
No.	Initials					per hr. per mm.²	per day
		years	*kg.*	*cm.*	mm.²	*cal.*	*cal.*
1	B.M.	20	54.12	167.0	1.60	32.60	1252
2	R.L.	20	61.98	167 6	1.70	31.67	1292
3	M.B.	23	58.87	158.4	1.61	35.66	1378
4	H.M.	21	56.45	172.7	1.67	31.89	1278
5	M.W.	19	54.68	159.7	1.56	33.09	1239
6	J.B.	19	57.90	159.4	1.59	32.65	1246
7	C.T.	20	61.20	164.5	1.72	34.62	1429
8	C.M.	20	75.15	167.3	1.85	31.19	1385
9	L.T.	22	55.60	166.4	1.61	31.11	1202

[1] Estimated by the height-weight formula of DuBois and DuBois ('16).

to 94%. All diets were supplemented with minerals (Ca, P, Na, Cl, Mg, K, Fe, Mn, Cu, I and S) and with the following amounts of vitamins per subject daily: 3 mg. of thiamine hydrochloride, 3 mg. of riboflavin, 15 mg. of nicotinamide, 1.5 mg. of pyridoxine and 2 mg. of calcium pantothenate. Each subject received daily 2 drops of haliver oil and 20 drops of solvent-extracted wheat germ oil.

The basal diet furnished from 0.14 to 0.23 gm. of nitrogen daily and consisted of sugar-cornstarch-lard cookies, supplemented with sucrose, lactose, fondant, jelly, butterfat, lemon juice, apple sauce, lettuce and French dressing. Water and table salt were allowed ad libitum. The test foods replaced isocaloric amounts of some of the nitrogen-free constituents of the basal diet at varying nitrogen levels. The diets were all consumed at caloric levels just adequate to maintain a constant body weight. The estimated physiological fuel value required ranged from 1.6 to 2.4 times the basal energy expenditure, averaging about 2.0 times. No attempt was made to standardize the proportion of food calories de-

rived from fat, which varied rather widely in individual periods but for the different foods averaged from 26 to 42%. This circumstance did not appear to contribute to the variability of the data secured. Farr ('39) has shown that a five-fold variation in the fat intake of two children with the nephrotic syndrome on isocaloric diets had no appreciable effect on the nitrogen balance.

The protein foods tested consisted of soy flour,[3] patent white flour,[4] milk,[5] a soy-white flour combination containing 13% of the former, supplying about 36% of the nitrogen of the mixture, and a typical mixture of foods modified from list 1 proposed by the Food and Nutrition Board of the National Research Council in Circular no. 115 issued in January, 1943. The latter mixture was consumed in three meals according to the following menu (all quantities in grams): Breakfast: oranges 120, oatmeal 27, egg 43, milk 229; luncheon: tomato soup 240, lettuce 60, peaches 120, soy wheat biscuits 48, milk 200; dinner: ground beef 65, beef suet 10, potatoes 240, carrots 120, cabbage 80, pineapple 24, soy wheat biscuit 48. For the purpose of testing the protein value of this mixture of foods, they were fed in three periods to the subjects participating in the tests in amounts equal to one-half, three-eighths and one-fourth of the quantities given above. The individual foods were also tested at different levels. All test foods, in this combination and among the individual foods, were cooked in some appropriate manner if they are commonly prepared by cooking. The milk was pasteurized. The oranges, lettuce, carrots and cabbage were served raw, and the peaches, pineapple and tomato soup were canned. The experimental meals were prepared and served in the Nutrition Laboratories of the Department of Home Economics. All test foods were analyzed for nitrogen, moisture, and ether extract by the approved methods of the A.O.A.C., and their heats of combustion (gross energy) were determined by the Parr oxygen-bomb calorimeter. The constituents of the basal diet containing appreciable amounts of nitrogen were also analyzed for this element, but their contents of water, fat and carbohydrate were estimated from tables of food composition (Chatfield and Adams, '40).

The test periods were continued until the nitrogen output in the urine, determined daily, showed no progressive change and was reasonably constant for 3 or more days. The average length of the periods was

[3] Staley's Stoy flour, containing 7.67% nitrogen.

[4] Pillsbury's enriched white patent flour, containing 2.05% nitrogen.

[5] Four per cent homogenized, pasteurized milk from the University creamery, containing an average of 0.553% nitrogen.

7.6 days, though there was considerable variation depending mainly on the type of diet consumed in the prior period.

The average daily output of nitrogen in the urine for the last 3 or 4 days of the period, or sometimes longer up to 9 days, was used in computing the nitrogen balance characteristic of the source and level of nitrogen consumed. The feces were collected for periods of 3 to 5 days and were marked off by either Fe_2O_3 or Cr_2O_3, given in 0.3 to 0.5 gm. doses. They were preserved by refrigeration and then homogenized with dilute acetic acid in a Waring Blendor.

Either four or five subjects participated in the study of each test food and for each test food there were from 7 to 13 experimental periods, making a total of 48 periods for all foods. In addition, two of the Subjects, nos. 3 and 4, completed tests on the low-nitrogen basal diet.

THE EXPERIMENTAL RESULTS

The most significant information afforded by these experiments concerns the relationship between the nitrogen intake and the nitrogen balance for the different sources of dietary nitrogen. By expressing each of these variables in milligrams per basal calorie, it is possible to pool the data for all subjects regardless of body size (Smuts, '35). This has been done in the respective graphs of figure 1. All of the individual data are plotted in these graphs, the small number beside each circled point referring to the subject supplying the measurement. The two experimental periods on the basal low-nitrogen diet for Subjects 3 and 4 are included in each graph.

The rectilinearity of the relationship between nitrogen intake and nitrogen balance for the same dietary source of nitrogen seems obvious from all charts, and is particularly evident from those for milk, white flour, and the combination of soy and white flour. Hence, equations representing straight lines of the type $Y = a + bX$ were fitted to each of the five sets of data by the method of least squares. In these equations, Y represents the nitrogen balance in milligrams per basal calorie; X represents the nitrogen intake expressed in the same terms; *a* represents the intercept of the line on the Y axis, or the total nitrogen loss in milligrams per basal calorie when the nitrogen intake is zero; and *b* defines the slope of the line, or the change in nitrogen balance in milligrams per milligram of change in nitrogen intake.

The *a* values in the five equations range from 1.719 to 1.924, and average 1.830 mg. per basal calorie. This value represents the total loss of nitrogen from the body on a nitrogen-free diet, expressed in milligrams per basal calorie. In the two periods with Subjects 3 and 4 subsisting

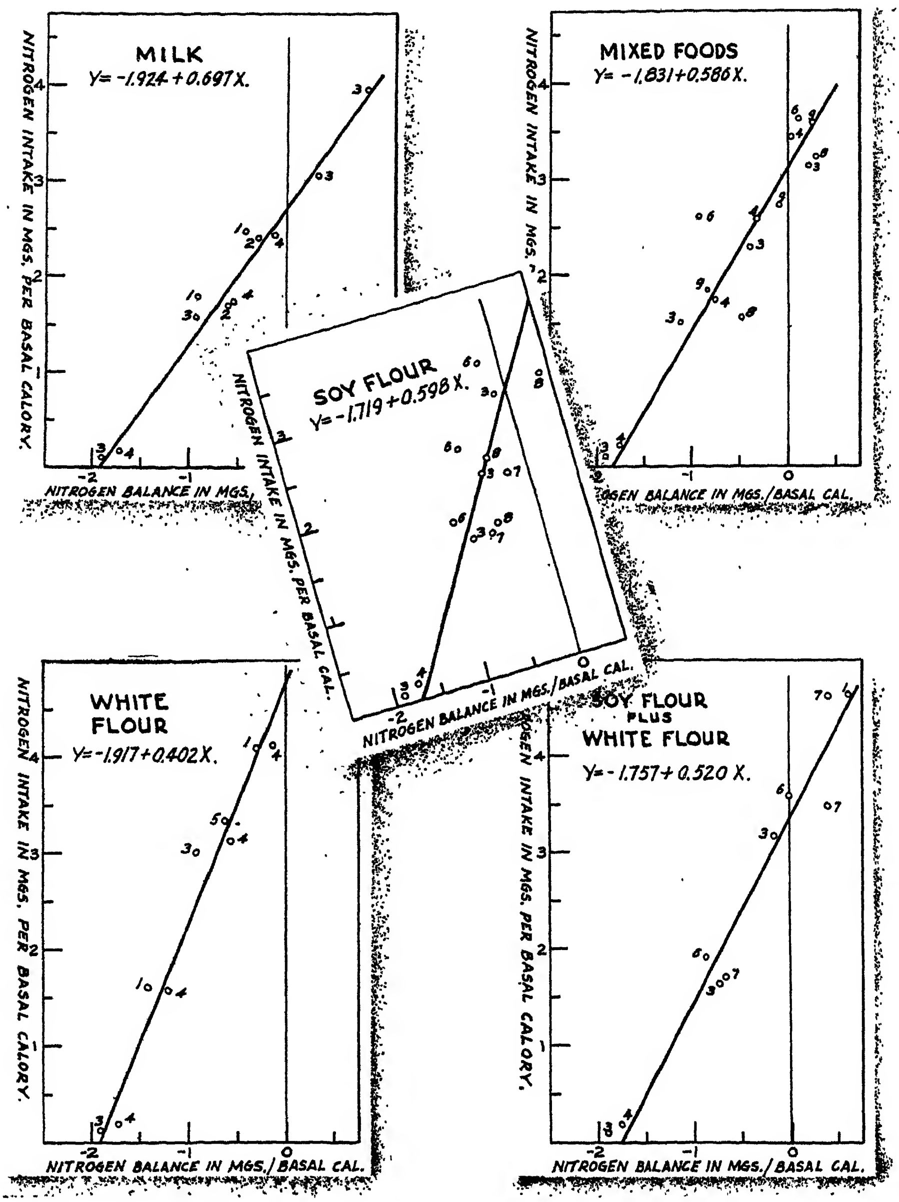


Fig. 1 The relationship between nitrogen intake and nitrogen balance on different diets. The $Y = -a + bX$ equations define the relationship for the various foods, the *a* value representing the nitrogen excretion at *0* intake and the *b* value the slope of the line, or the product of the biological value and the true digestibility of the nitrogen. The numbers 1–9 adjacent to the circled points refer to the subjects yielding the data from which the calculated curves were drawn.

on a practically nitrogen-free diet, the fecal (metabolic) nitrogen amounted to 0.410 and 0.401 mg. per basal calorie, an average of 0.405. If this value is deducted from 1.830, the difference, 1.425, is the average minimum endogenous urinary nitrogen in milligrams per basal calorie.[6]

The *b* values in the five equations define the slope of the lines describing the respective relationships between the nitrogen intake and the nitrogen balance, both expressed in milligrams per basal calorie. Each value is the product of the coefficient of true digestibility computed in the usual manner and the biological value in the Thomas ('09) sense, each expressed as a decimal rather than as a percentage.[7] The *b* values with their standard errors[8] are as follows: for milk nitrogen, 0.697 ± 0.042; for the nitrogen of white flour, 0.402 ± 0.027; for the nitrogen of soy flour, 0.598 ± 0.086; for the nitrogen of the soy-white flour combination, 0.520 ± 0.046; and for the nitrogen of the mixed food combination, 0.586 ± 0.059. Thus, the average over-all nutritive quality of the different sources of dietary nitrogen ranges as follows in the order of decreasing average quality: milk, soy flour, mixed foods, soy flour plus white flour, and white flour.

The statistical significance of the differences in *b* values have also been computed with the results summarized in table 2. If the probabili-

[6] Smuts ('35) found an average of 2 mg. of endogenous urinary nitrogen per basal calorie for a series of mice, rats, guinea pigs, rabbits and pigs, mainly adult males, and from the meager information available concluded that approximately the same ratio applied to men. However, the possibility of a sex difference in this respect was not considered. Palmer, Means and Gamble ('14) observed a marked sex difference in the ratio of urinary creatinine excretion to basal metabolism, such that for men 1.02 mg. of creatinine were excreted per basal calorie, while for women the ratio was only 0.8 as much. If it may be assumed that this sex difference applies to the total output of minimal endogenous urinary nitrogen, the Smuts' ratio of 2 mg. per basal calorie for men would correspond to a ratio of 1.6 mg. per basal calorie for women. This value is not greatly different from that of 1.425 estimated for the women subjects of this experiment.

[7] The subscripts to N in the following equations relate to the different factors entering into a nitrogen metabolism study, N standing for nitrogen in all cases: b = balance, i = intake, f = fecal, u = urinary, m = metabolic fecal, and e = minimum endogenous urinary. In these terms the regression line relating nitrogen balance to nitrogen intake becomes —
$N_b = -a + bN_i$, in which $a = N_m + N_e$ and $N_b = N_i - N_f - N_u$.

Substituting and rearranging — $b = \dfrac{N_i - (N_f - N_m) - (N_u - N_e)}{N_i}$.

The coefficient of true digestibility of nitrogen, in these terms, would be equal to $\dfrac{N_i - (N_f - N_m)}{N_i}$, and dividing this into b —

$$b \cdot \frac{N_i}{N_i - (N_f - N_m)} = \frac{N_i - (N_f - N_m) - (N_u - N_e)}{N_i - (N_f - N_m)} = \text{the biological value.}$$

[8] The standard errors were computed according to the method of Rider ('39, pp. 93, 94), as well as the statistical significance of the differences in *b* values.

ties (last column of this table) that a random combination of the uncontrolled factors in the experiment would by themselves produce differences as great or greater than those observed are as small or smaller than 0.030, they may reasonably be neglected. In such cases, it may be concluded that the differences are real, being due to actual differences in the over-all nutritive qualities of the sources of dietary nitrogen compared. On the basis of this reasoning, it may be concluded that milk nitrogen is definitely superior to the nitrogen of white flour and of soy flour plus white flour in the combination used. Patent white flour is definitely inferior to all other sources of dietary nitrogen tested. No difference in over-all nutritive quality has been demonstrated in any of the other comparisons, although the data suggest the superiority of milk nitrogen over that of the mixed foods. However, since milk nitrogen is definitely better than the nitrogen in the combination of soy flour and white flour, while neither soy flour nitrogen nor the nitrogen of the mixed foods has been so characterized, it would seem reasonable to interpret the entire picture as establishing the following three classes of dietary nitrogen:

TABLE 2

Statistical significance of differences in *b* values.

FOODS COMPARED	DIFFERENCE IN *b* VALUES	n	t	P
Milk and white flour	.295	16	5.931	< .005
Milk and soy flour	.099	20	1.036	ca. .15
Milk and soy + white flour	.177	17	2.705	.008
Milk and mixed foods	.111	22	1.417	.088
White flour and soy flour	.196	18	2.200	.022
White flour and soy + white flour	.118	15	2.077	.029
White flour and mixed foods	.184	20	2.618	.0086
Soy flour and soy + white flour	.078	19	.818	> .15
Soy flour and mixed foods	.012	21	.114	> .15
Mixed foods and soy + white flour	.066	21	.881	> .15

(1) Milk; (2) soy flour, soy flour and white flour, mixed foods; and (3) white flour. This statement does not imply that real differences do not exist among the foods of Class 2. The variability of the data was such that differences have not been established statistically.

If the nitrogen of soy flour and that of white flour exerted no mutual supplementary effects when combined in the proportion of 36 parts of the former to 64 parts of the latter, then the *b* value of the combination would be 0.473. However, the statistical significance of the difference between this value and that observed for this combination, i.e., 0.520,

cannot be established, mainly because of the large standard error of the b value of soy flour. The data suggest the existence of a supplementary relation.

The fecal nitrogen in these experiments seemed to be largely of body, rather than of food, origin. When expressed per gram of dry matter consumed, there was no demonstrable correlation with the nitrogen intake, that is, with the nitrogen content of the dry matter consumed. Furthermore, the average amounts of fecal nitrogen in milligrams per gram of dry matter consumed varied within a small range for all test diets, the averages with their standard errors being as follows: milk, 1.18 ± 0.21; white flour, 1.29 ± 0.13; soy flour, 1.42 ± 0.17; mixture of white and soy flour, 1.37 ± 0.12; and mixed foods, 1.78 ± 0.13. In the two periods on the low-nitrogen basal diet, the fecal nitrogen amounted to 0.91 mg. per gram of dry matter consumed for Subject 4, and 1.14 mg. for Subject 3.

The average coefficients of apparent digestibility of the nitrogen in the different foods and food combinations tested were: 77.2 for milk, 81.6 for white flour, 70.3 for soy flour, 77.9 for the combination of soy and white flour, and 75.8 for the mixed foods.

The true digestibility of the nitrogen of the foods and combinations tested can be computed by assessing the metabolic nitrogen in the feces at 1 mg. per gram of dry matter consumed, this value being the average of the two values obtained with Subjects 3 and 4 on the basal low-nitrogen diet, 0.908 and 1.140. The true digestibilities thus computed are as follows: 94 for milk, 97 for white flour, 92 for soy flour, 94 for the combination of soy and white flour, and 90 for the mixed foods. Because of the large amounts of foods in all of the experimental diets contributing practically no nitrogen to the intake, but inducing an excretion of nitrogen in the feces in proportion to the amounts of dry matter they contain, these coefficients of true digestibility are of greater significance than the coefficients of apparent digestibility given in the preceding paragraph.

The amounts of nitrogen required for equilibrium may be computed from the equations relating intake and balance of nitrogen for the various test foods by equating the balance (Y) to zero and solving for X. The values thus obtained are summarized in table 3 for the various foods and food combinations tested, together with other information pertinent to the nutritive evaluation of their protein components.

The values given in the last two columns of this table are the most significant results secured in these experiments, defining as they do the amounts of nitrogen and of conventional protein required for mainte-

nance (as ordinarily determined) in the adult organism. The nitrogen requirement for the most general use is expressed in milligrams per basal calorie. This method of expression takes account of differences in body size more effectively than the customary method relating requirement to body weight.

While on the white flour diet providing 6.819 gm. of nitrogen daily, Subject 3 excreted 6.813 gm. of nitrogen daily in the urine during a 5-day period and was in negative nitrogen balance to the extent of 0.872 gm. daily. For the next 5 days she consumed essentially the same diet plus a daily supplement of lysine, providing 0.263 gm. of nitrogen.

TABLE 3

The nutritive value of the nitrogen components of the foods and food combinations tested.

TEST FOODS	TRUE DIGESTIBILITY PCT.	BIOLOGICAL VALUE [2] PCT.	REQUIRED FOR EQUILIBRIUM	PROTEIN ($N \times 6.25$) REQUIRED PER DAY [3]
			mg. per basal cal.	*gm.*
Milk	94.0	74	2.760	22.4
White flour	96.9	41	4.763	38.7
Soy flour	91.9	65	2.876	23.4
Soy-white flour [1]	94.2	55	3.380	27.5
Mixed foods	89.6	65	3.124	25.4

[1] Thirty-six parts of soy bean protein to 64 parts of white flour protein.

[2] Computed from the respective *b* value in the equation expressing the relation between N intake and N balance, by dividing by the true digestibility expressed as a decimal.

[3] For a basal metabolism of 1300 cal. daily, the average for the nine subjects of this experiment.

Immediately the urinary nitrogen dropped by almost 0.9 gm. daily and the subject went into positive balance. This result confirms the experience of Rose ('44) and of Albanese et al. ('41) that the adult human requires lysine, and brings out a difference in this respect between adult human and adult rodent nutrition (Burroughs, Burroughs and Mitchell, '40).

DISCUSSION

The biological values for the nitrogen in the foods and food combinations tested, given in table 3, may be compared with the few others reported in the literature, although there is no high degree of agreement. For milk protein, Sumner and Murlin ('38) report a biological value of 62, Lintzel and Bertram ('38) one of 43, and Martin and Robison ('22) one of 51, for adult humans, compared with our value of 74. Wagner ('23) obtained a value for milk proteins of 60 with a group of children with latent hilus gland tuberculosis. The differences are largely unex-

plainable, although in some cases, it may be questioned whether the true minimal endogenous urinary nitrogen was ever attained, or whether the caloric intake was adequate (Lintzel and Bertram, '38). In the present experiments the biological values given relate to a definite caloric intake, that required for energy equilibrium. A larger, or a smaller, caloric intake may be expected to change the picture by sparing dietary protein to a greater or lesser extent, as Neumann ('19) and Cuthbertson and Munro ('37, '39) have shown. It would seem that the most significant biological values for the adult human would relate to energy intakes just sufficient to maintain the body weight.

For soy proteins, an average biological value of 65 was reported for soybean curd by Cheng, Li and Lan ('41), comparing favorably with the value of 65 for soy flour given in table 3. The high value of 81 for soybean protein reported by Murlin et al. ('44) may probably be explained by the short low-nitrogen period of 3 days, during which time the minimal level of urinary nitrogen may not have been established.

The nitrogen requirements for equilibrium given in table 3 may be computed in terms of conventional protein ($N \times 6.25$) for the conventional body weight of 70 kilograms. Such a person would have a surface area of about 1.8 square meters and a basal metabolism of approximately 1650 cal. per day. Hence, his daily requirement of protein from the sources tested would be: from milk, 28.5 gm.; from white flour 49.1 gm.; from soy flour, 29.7 gm.; from soy flour 13 parts and white flour 87 parts, 34.9 gm.; and from the mixed diet, 32.2 gm. If the ratio of minimum endogenous urinary nitrogen to basal calories is less in women than in men, as the results of Palmer, Means and Gamble ('14) may suggest, the above values should be increased by about 18%.[9]

The above estimates of protein needs in adult life assume that the nitrogen output from the body is composed solely of the urinary and fecal output, and that the protein requirements of adulthood are completely covered when the body is in nitrogen equilibrium. Holt ('44) has pointed out certain possible errors in this latter assumption, but the bases of his arguments need either confirmation or experimental proof. In any case their applicability to the situation under discussion is doubtful. However, it is undoubtedly true that throughout adult life some tissues are continually growing. Hrdlička ('36) has called attention to this fact with reference to stature, various head and face dimensions, the chest, hands and feet. But aside from these growth changes in body size and dimension, it is known that certain tissues of the body, such as the epidermis and epidermal structures, grow continuously. All

[9] Assuming no sex difference in the ratio of fecal metabolic nitrogen to dry matter consumed.

these growth changes involve protein, so that the adult, to maintain the body in its normal and proper nutritive status, must receive more dietary protein than that required merely for equilibrium. What this excess should be has not been assessed insofar as the authors are aware.

Grindley ('12) has reported continuous nitrogen balance studies carried out over a period of 220 days on 23 adult men from 18 to 31 years of age. They were consuming an adequate diet, containing 80 to 85 gm. of protein daily, and body weight variations were slight. The daily nitrogen balance averaged 1.38 gm., with a coefficient of variation of 22.3. The balance seemed to be appreciably correlated with body surface ($r = +0.480$), and per square meter averaged 0.77 gm., with a coefficient of variation of 19.3. This apparent storage of nitrogen by adult men can be broken down into two factors; one, a failure to measure all of the nitrogen output from the body, specifically that from the skin; and, two, a true retention of nitrogen in the growth of hair, epidermal tissues and structures, and in the changes in size and form of the body characteristic of the later phases of growth.

In unpublished experiments, Mitchell and Hamilton have measured the dermal losses of nitrogen in four adult men while at comparative rest in an air-conditioned room. Under comfortable, non-sweating conditions this loss averaged 0.40 gm. daily; under profuse sweating conditions it averaged 3.74 gm. Applying the former figure to the average positive balance of nitrogen observed in Grindley's subjects, it would appear that approximately 1 gm. of nitrogen a day $(1.38 - 0.40)$ was actually stored in the bodies of these adult men.

An estimate of the total requirement of nitrogen by the women subjects of this experiment on the basis of the information considered above may be made by computing, from the equations relating nitrogen intake to nitrogen balance for each of the test foods, that intake of nitrogen required to induce a storage of 0.77 gm. of nitrogen per square meter of body surface. The average surface area of the subjects in this study was 1.66 $mm.^2$ and their average basal metabolism was 1300 cal. per day. Hence, the above nitrogen storage, in milligrams per basal calorie, is $(0.77 \times 1.66) \div 1300 = 0.983$ mg. The amounts of protein of the various species tested required for maintenance and "adult growth" are given in table 4.

The requirements here computed are averages relating to a nutritional status characteristic of the subjects of this experiment. For adult women in general, such requirements in all probability exhibit considerable variation. Individuals may be able to adapt themselves to lower planes of protein nutrition (Mitchell, '44), by diminishing the

inevitable wastage of nitrogen in the endogenous metabolism, or by making better use of the protein supply available through the protein-sparing action of a more abundant intake of carbohydrates.

The differences in requirement depending upon the source of the dietary protein are great. It is interesting to note that the estimated protein requirement on a mixed diet is among the lowest of those secured.

TABLE 4

The amounts of nitrogen and of conventional protein required for true maintenance and "adult growth".

SOURCE OF NITROGEN	NITROGEN REQUIRED PER BASAL CAL.[1]	PROTEIN (N × 6.25) REQUIREMENT	
		Average for the subjects of this experiment	Per 70 kg. body weight
	mg.	*gm. per day*	*gm. per day*
Milk	4.171	33.9	43.0
White flour	7.214	58.6	74.4
Soy flour	4.518	36.7	46.6
Soy-white flour combination	5.269	42.8	54.3
Mixed foods	4.802	39.0	49.5

[1] These figures were obtained by substituting + 0.983 mg. N for the Y in the respective Y = a + bX equations, and solving for X.

SUMMARY AND CONCLUSIONS

Fifty nitrogen balance periods were carried out upon nine women subjects to determine the requirements for protein during adult life for different types of dietary protein. The protein foods tested were milk, soy flour, white flour, a combination of soy flour and white flour containing 13% of the former, and a well balanced mixture of protein foods. All test foods were cooked in some appropriate manner if they are commonly prepared by cooking. The test foods supplied 91% or more of the total protein in the test diets, which were fed in amounts to maintain body weight.

Each food, or food combination, was studied at different levels of nitrogen intake in order to define the relationship between intake and balance. This relationship was shown to be rectilinear and satisfactorily described by the equation of a straight line in which nitrogen balance is the dependent variable (Y). The slope of the line, the *b* constant in the equation, is the product of the true digestibility of the dietary nitrogen and its biological value in the Thomas sense. The intercept of the line on the ordinate representing zero balance gives the amount of dietary nitrogen required for equilibrium. In these computations, all values for the different subjects are pooled together for each

test food by expressing them in milligrams of nitrogen per calorie of basal heat.

For milk, white flour, soy flour, the soy-white flour combination and the mixed foods, the indicated biological values were, respectively, 74, 41, 65, 55 and 65, and the amounts of nitrogen required for equilibrium (as ordinarily measured) were, respectively, 2.76, 4.76, 2.88, 3.38, and 3.12 mg. per basal calorie.

When due allowance is made for probable dermal losses of nitrogen and for the growth of tissues during adult life, the average daily requirements of conventional protein ($N \times 6.25$) calculated to a weight of 70 kg., a surface area of 1.8 square meters and a basal metabolism of 1650 cal. daily, are: 43 gm. for milk, 74 gm. for white flour, 47 gm. for soy flour, 54 gm. for the soy-white flour combination, and 50 gm. for the mixed foods. The limited significance of these numerical values is pointed out.

Some evidence is presented that the human adult, unlike the adult rat, requires lysine for nitrogen equilibrium.

LITERATURE CITED

ALBANESE, A. A., L. E. HOLT, JR., J. E. BRUMBACK, JR., M. HAYES, C. KAJDI AND D. M. WAGERIN 1941 Nitrogen balance in experimental lysine deficiency in man. Proc. Soc. Exp. Biol and Med., vol. 48, p. 728.

BURROUGHS, E. W., H. S. BURROUGHS AND H. H. MITCHELL 1940 The amino acids required for the complete replacement of endogenous losses in the adult rat. J. Nutrition, vol. 19, p. 363.

CHATFIELD, C., AND G. ADAMS 1940 Proximate composition of American food materials. U. S. Dept. of Agr. Circ. no. 549, pp. 91.

CHENG, L. T., H. C. LI AND T. H. LAN 1941 Biological values of soybean protein and mixed soybean-pork and soybean-egg proteins in human subjects. Chinese J. Physiol., vol. 16, p. 83.

CUTHBERTSON, D. P., AND H. N. MUNRO 1937 A study of the effect of overfeeding on the protein metabolism of man. III. The protein-saving effect of carbohydrate and fat when superimposed on a diet adequate for maintenance. Biochem. J., vol. 31, p. 694.

——— 1939 The relationship of carbohydrate metabolism to protein metabolism. I. The roles of total dietary carbohydrate and of surfeit carbohydrate in protein metabolism. Biochem. J., vol. 33, p. 128.

DUBOIS, D., AND E. F. DUBOIS 1916 Clinical calorimetry. Tenth paper. A formula to estimate the approximate surface area if height and weight be known. Arch. Intern. Med., vol. 17, p. 863.

FARR, LEE E. 1939 Assimilation of protein by young children with the nephrotic syndrome. II. Effect of dietary fat and carbohydrate on nitrogen balance. Am. J. Diseases Children, vol. 58, p. 935.

GRINDLEY, H. S. 1912 Studies in nutrition. An investigation of the influence of saltpeter on the nutrition and health of man with reference to its occurrence in cured meats. Vol. IV. The experimental data of the biochemical investigations. University of Illinois, 494 pp.

HARRIS, L. E., AND H. H. MITCHELL 1941 The value of urea in the synthesis of protein in the paunch of the ruminant. I. In maintenance. J. Nutrition, vol. 22, p. 167.

HOLT, L. EMMETT, JR. 1944 Amino acid deficiencies in man. ''Implications of Nutrition and Public Health in the Postwar Period''. Detroit, Michigan, p. 193.

HRDLIČKA, ALEŠ 1936 Growth during adult life. Proc. Am. Philosophical Soc., vol. 76, p. 847.

LEITCH, I., AND J. DUCKWORTH 1937 The determination of the protein requirements of man. Nutr. Abst. and Revs., vol. 7, p. 257.

LINTZEL, W., AND W. BERTRAM 1938 Experimentelle Studien zur Theorie des Eiweissstoffwechsels. II. Die biologische Wertigkeit des Milcheiweisses beim Erwachsenen. Biochem. Zeit., vol. 297, p. 315.

MARTIN, C. J., AND ROBERT ROBISON 1922 The minimum nitrogen expenditure of man and the biological value of various proteins for human nutrition. Biochem. J., vol. 16, p. 407.

MELNICK, D., AND G. R. COWGILL 1937 The protein minima for nitrogen equilibrium with different proteins. J. Nutrition, vol. 13, p. 401.

MITCHELL, H. H. 1944 Adaptation to undernutrition. J. Am. Diet. Assn., vol. 20, p. 511.

MURLIN, J. R., L. E. EDWARDS AND E. E. HAWLEY 1944 Biological values and true digestibilities of some food proteins determined on human subjects. J. Biol. Chem., vol. 156, p. 785.

NEUMANN, R. R. 1919 Die ''Kriegsernährung in Bonn im Winter 1916–1917 auf Grund experimenteller Untersuchung. Vierteljahrschr. f. gericht. Med., vol. 57, p. 1.

PALMER, W. W., J. H. MEANS AND J. L. GAMBLE 1914 Basal metabolism and creatinine elimination. J. Biol. Chem., vol. 19, p. 239.

RIDER, P. R. 1939 An Introduction to Modern Statistical Methods. New York, pp. 229.

ROSE, M. S., AND G. MACLEOD 1925 Maintenance values for the proteins of milk, meat, bread and milk, and soy bean curd. J. Biol. Chem., vol. 66, p. 847.

ROSE, W. C. 1944 The role of protein in the diet. Proc. Inst. Med. of Chicago, vol. 15, p. 24.

SHERMAN, H. C., J. C. WINTERS AND V. PHILLIPS 1919 Efficiency of oat protein in adult human nutrition. J. Biol. Chem., vol. 39, p. 53.

SMUTS, D. B. 1935 The relation between the basal metabolism and the endogenous nitrogen metabolism, with particular reference to the estimation of the maintenance requirement of protein. J. Nutrition, vol. 9, p. 403.

SUMNER, E. E., AND J. R. MURLIN 1938 The biological value of milk and egg protein in human subjects. J. Nutrition, vol. 16, p. 141.

SUMNER, E. E., H. B. PIERCE AND J. R. MURLIN 1938 The egg-replacement value of several proteins in human nutrition. J. Nutrition, vol. 16, p. 37.

THOMAS, K. 1909 Ueber die biologische Wertigkeit der Stickstoffsubstanzen in verschiedenen Nahrungsmittel. Arch. f. Anat. u. Physiol., Physiol. Abt., p. 219.

WAGNER, R. 1923 Zur biologischen Wertigkeit der stickstoffhaltigen Nahrungsmittel. Ein Beitrag zum minimum Gesetz in der Ernährungslehre (Nach Versuchen am wachsenden Organismus). Zeit. f. d. ges. exp. Med., vol. 33–34, p. 250.

THE EFFECTS OF LIGHT, TRAUMA, RIBOFLAVIN, AND ARIBOFLAVINOSIS ON THE PRODUCTION OF CORNEAL VASCULARITY AND ON HEALING OF CORNEAL LESIONS [1]

OLIVER H. LOWRY AND OTTO A. BESSEY
Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York

(Received for publication June 4, 1945)

The growth of blood capillaries from the limbic plexus into the cornea has been observed as a response of this normally avascular tissue to a variety of adverse conditions (Duke-Elder, '42; Wolbach and Bessey, '42; Nutrition Reviews, '43); i.e., physical and chemical trauma, infections, and certain experimental dietary deficiencies. Among the dietary deficiencies, corneal vascularity has been observed in experimental vitamin A deficiency, ariboflavinosis, and in deficiencies of sodium, zinc, and the amino acids lysine and tryptophane. It has been clearly established that in man, just as in experimental animals, a deficiency of riboflavin may lead to an invasion of the cornea by capillaries (Sydenstricker, Sebrell, Cleckley and Kruse, '40). As a result, some investigators have attributed the mild degree of corneal vascularity found among apparently normal population groups, as well as among less well nourished groups, to riboflavin deficiency (Wiehl and Kruse, '41; Tisdall, McCreary and Pearce, '43; National Research Council, '43). This mild lesion has been found to be highly prevalent, particularly among sailors, aviators, and others exposed as a result of their activities to excessive sunlight; e.g., at high altitudes, on the desert, or from snow reflection of light or reflection from the water (unpublished confidential information). Since riboflavin is rapidly destroyed by light, the possibility exists that the exposure of the eye to bright light might further limit the riboflavin in the cornea and thereby accentuate the growth of capillaries into this structure. Bright light might even induce

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Public Health Research Institute of The City of New York, Inc.

a local riboflavin deficiency in the cornea in spite of an adequate intake of the vitamin for the rest of the body.

Trauma, if severe enough, also induces the growth of vessels into the cornea (Duke-Elder, '42). Ariboflavinosis might enhance this invasion of the cornea or delay recovery from the trauma. It is a common clinical experience that injuries to the cornea heal more quickly if the eye is bound so as to exclude light. It is conceivable that this benefit obtains through protection of the corneal riboflavin from destruction by light. If this were the case, a high riboflavin intake would be expected to overcome some of the detrimental effects of bright light on the healing of corneal injuries.

To study these effects and possibilities, an investigation has been made of the influence of light on the development of corneal lesions in riboflavin deficiency, and the influence of light and riboflavin on the rate of healing of injuries to the cornea. Three types of experiments have been made:

1. The rate of capillary ingrowth and other corneal changes have been compared in four groups of rats maintained under the following conditions: (a) Normal diet, kept in the dark; (b) normal diet, kept in continuous light; (c) riboflavin deficient diet, kept in the dark; (d) riboflavin deficient diet, kept in continuous light.

2. The rate of repair of injuries to the cornea produced by ultraviolet light, removal of the corneal epithelium, or by nitric acid has been assessed in groups of rats kept under the following conditions: (a) Normal diet, kept in the light; (b) normal diet, kept in the dark; (c) riboflavin deficient diet, kept in the light; (d) riboflavin deficient diet, kept in the dark; (e) normal diet plus extra riboflavin, kept in the light; (f) normal diet plus extra riboflavin, kept in the dark.

3. Observations have been made on the effects of feeding extra riboflavin on the prevalence of spontaneous corneal vascularity which regularly occurs in supposedly normal stock rats.

EXPERIMENTAL

Albino rats of the Wistar strain, 45 to 60 gm. in weight at the start of the experiment, were used in all experiments except for the ultraviolet irradiation injury; in this case the animals weighed 125–150 gm. The diet, supplements, and method for maintaining the animals in continuous light have been previously described (Bessey and Lowry, '44). Observations of the cornea were made with a binocular dissecting microscope using 3 × objectives and 10 × oculars. The cornea was illuminated with a 16 amp. 6-volt Mazda microscope illuminator lamp with

the image of the filament focussed on the eye ball. The light beam was allowed to pass through a nickel chloride solution to remove most of the heat. The beam of light was horizontal and the limbus was observed from above. By turning the eye so that it looked directly at the light, and allowing the light to fall on the lower portion of the cornea, the eye ball was filled with light and the limbic capillaries became easily visible against a brilliant white background. Small rats were held unanesthetized in the proper position with the eye ball protruding through tension with the fingers on either side of the eye. Larger rats were lightly anesthetized with ether. In some cases the limbic vessels were more easily observed after dilatation of the pupil with 2% homatropine. The normal vessels which extend only 0.25 to 0.4 mm. into the cornea were, however, more easily observed without mydriasis. The length of the capillaries was measured by comparison with the separation between hairs placed in the ocular of the microscope. The vessels were in some instances photographed by mounting a camera (Loveland, '43) over one eye piece of the binocular microscope and judging the focus through the other eye piece. When the rat was quiet and the vessels exactly in focus, the camera shutter was tripped with a foot attachment.

The three types of trauma used in studying the corneal response to injury were produced in the following way: The corneal epithelium was removed with sand paper. It was difficult to avoid some injury to the underlying stroma as well. With damage to the stroma, healing was greatly delayed. This introduced a factor difficult to control. The trauma from acid was produced by applying nitric acid, 1N or 5N, for a definite length of time with a wooden applicator which had been reduced to about ½ mm. in diameter and which was provided with a thin cotton wrapping to give a total diameter of about 1 mm. By dipping this applicator in the diluted nitric acid and applying it at right angles to the cornea, a damaged area 1 mm. in diameter could be produced. The irradiation with ultraviolet light was accomplished by anesthetizing the rats with Nembutal, binding one eye open with adhesive tape and exposing the open eye to the light from a General Electric H4 mercury vapor lamp. The head of the rat was bound to a board with a strip of adhesive tape held in place with thumb tacks. In this way the cornea was kept at a standard distance (5 cm.) from the envelope of the lamp. Of the three types of trauma produced, this last was the most reproducible.

The changes resulting from riboflavin deficiency were grouped according to (a) length of corneal capillaries; (b) alteration of the capillaries, i.e., enlargement, bulging and sprouting; (c) turbidity of the cornea,

and (d) swelling and redness of the conjunctiva. The changes, other than change in length of capillaries, were assigned numerical values as per cent of the severest changes observed; e.g., slight, moderate, severe, and extreme changes were recorded as 25, 50, 75, and 100%, respectively. The values for "capillary alteration" represent the average of observed values for enlargement, bulging and sprouting. In the case of trauma to the eye, the changes were assessed in an analogous manner, complete healing of the cornea being considered as 100% recovery.

RESULTS AND DISCUSSION

Influence of light on the corneal vascularization of ariboflavinosis

In general, long exposure to light from incandescent lamps failed to accelerate the invasion of the cornea by blood vessels in ariboflavinosis. At the end of 28 days (table 1) rats on a riboflavin-free diet were no less severely affected if maintained in darkness than if kept in bright light.

In the normal rat, capillaries extend 0.25 to 0.4 mm. into the cornea from the limbic vessels. In the event of severe riboflavin deficiency, the capillaries will grow to many times their initial length. It is unusual to

TABLE 1

Observation of eyes after 28 days on experiment.

DIET	ENVIRONMENT	NO. OF ANIMALS	INITIAL WEIGHT	GAIN IN 28 DAYS	CORNEA: Capillary length	CORNEA: Capillary alteration	CORNEA: Turbidity	CONJUNCTIVA: Swelling and redness
			gm.	*gm.*	*mm.*	*% of maximal* [1]		
Riboflavin deficient	Dark	8	58	8	0.9	45	38	50
Riboflavin deficient	Bright light	7	55	19	0.6	20	22	50
Normal control 20 μg. riboflavin daily	Dark	6	60	30	0.4	2	0	15
Normal control 20 μg. riboflavin daily	Bright light	7	55	55	0.4	7	2	20

[1] The derivation of these average percentage figures is based on an arbitrary percentage scale and these values should not be taken to indicate that the measurements could be made with great precision (see text).

observe growth limited to 0.1 or 0.2 mm. Thus, the average capillary lengths in the deficient corneas (table 1) represent the mean of a number of 0.3 or 0.4 mm. values with other values of 0.8 to 2 mm. In the control group no influence of light on the eyes could be observed at the end of 28 days, although early in the experiment the rats exposed to the brilliant illumination showed a moderate degree of reddening and swelling of the conjunctivae. Both groups of rats gained less weight in darkness than they did in the light. This depressant effect of nearly complete and continuous darkness has been observed previously (Dempsey). This growth effect appears to be without significance for the present studies since another group of rats maintained in moderate light developed eye changes on a riboflavin-free diet which were approximately the same as those shown in table 1.

These observations are in agreement with chemical analyses (Bessey and Lowry, '44) which demonstrated that light did not influence the concentration of riboflavin in the cornea. The chemical studies also showed that corneal changes were not visible until the riboflavin concentration in the cornea had fallen to less than half of normal. This would seem to indicate that the corneal changes are evidence of rather extreme depletion of riboflavin. This conclusion is supported by the prompt improvement of the cornea with very small doses of riboflavin.

Influence of light and riboflavin on the healing of corneal injuries

Exposure to ultraviolet light proved to be the most satisfactory form of trauma from the standpoint of reproducibility. The healing ability of the cornea was measured by the length of exposure which on repetition at 24-hour intervals would just produce mild but detectable changes in the cornea. The exposures were repeated as many as five or six times. The time of exposure required was reproducible within 15 or 20%.

With this method it could be easily shown (table 2) that (a) the cornea can tolerate more ultraviolet light if the intervals between exposures are spent in darkness; (b) that neither extra riboflavin nor 2 weeks' deprivation of riboflavin has demonstrable influence on the response to ultraviolet light; but (c) that riboflavin deprivation for longer periods in either the young or the adult does handicap the cornea in resisting trauma due to ultraviolet light, particularly if capillaries are already actively growing in the cornea.

Although the striking delay in the rate of healing due to light in all groups is consistent with the thesis that light might handicap the cornea by the local destruction of riboflavin, the chemical analyses of the cornea have failed to support this possibility (Bessey and Lowry, '44). Not

only did ultraviolet illumination alone, or exposure to incandescent lamps alone fail to decrease the corneal riboflavin concentration, but the combination of the two types of illumination was equally ineffective in lowering the riboflavin in the cornea. Although light exerts a striking effect, this does not seem explicable on the basis of riboflavin destruction. These observations indicate that although mild riboflavin deficiency does not lead to handicaps in healing of corneal injuries due to light, there is a degree of deficiency beyond which very definite handicaps result. Whether riboflavin deficiency is specific in this respect, or whether it is a response common to other deficiencies, cannot be decided without further studies. It does seem clear, however, that the effects from light and the effects from riboflavin deficiency are independent factors.

TABLE 2

Time of exposure to ultraviolet light required to produce definite corneal changes.

		NORMAL ADULTS FED		ADULTS RIBOFLAVIN DEFICIENT FOR		YOUNG RATS RIBOFLAVIN DEFICIENT	
		Normal diets [1]	Normal diets plus riboflavin [2]	17 days [3]	30 days	Quiescent capillaries	Growing capillaries
Rats kept in darkness between irradiation	Minutes required	28	28	25	15	18	12
	Number of animals	(19)	(6)	(5)	(4)	(4)	(2)
Rats kept in light between irradiation	Minutes required	10	10	10	..	..	..
	Number of animals	(8)	(4)	(4)	.	..	.

[1] Rats either on chow (3 μg. per gm. of diet) or purified diet plus 21 μg. riboflavin per day.
[2] Forty μg. extra riboflavin daily.
[3] No corneal changes visible before irradiation.

The other two types of corneal trauma employed, mechanical injury by removal of the epithelium and chemical injury with nitric acid, proved to be less uniform and less sensitive methods (table 3). Neither the light in itself nor the riboflavin deficiency alone retarded healing to a degree detectable by these methods. However, the combination of light after injury and riboflavin deficiency did lead to striking handicaps. The lack of sensitivity of these later methods may possibly be ascribed to the surface nature of the injuries compared with the presumably more penetrating characteristics of the injury caused by ultraviolet light. However, the possibility must be considered that light may

TABLE 3

Per cent recovery[1] of cornea 5 to 7 days after trauma.

	ADULTS KEPT IN DARK			ADULTS KEPT IN LIGHT		
	Normal[2]	Normal + riboflavin[3]	Riboflavin deficient[4]	Normal[2]	Normal + riboflavin[3]	Riboflavin deficient[4]
Removal of epithelium	60	68	68	50	68	0
Number of animals	(6)	(3)	(3)	(6)	(3)	(3)
5N HNO_3 for 15 seconds	52	32	50	72	50	18
Number of animals	(6)	(3)	(3)	(6)	(3)	(3)

[1] The derivation of these average percentage figures is based on an arbitrary percentage scale and these values should not be taken to indicate that the measurements could be made with great precision (see text).

[2] Three μg. riboflavin per gram of diet (chow) or about 30 μg. daily.

[3] Forty μg. extra riboflavin per day or a total of about 70 μg. daily.

[4] Thirty days on riboflavin deficient diet.

be more damaging to an injury produced by light than to other types of injury.

Influence of riboflavin on the development of "spontaneous" capillaries

Older rats on a dog chow diet frequently develop corneal vascularity which may occasionally approach the severity of invasion observed in riboflavin deficiency. To test the influence of added riboflavin on this "spontaneous" corneal vascularity, a group of twenty rats were put on a chow diet at the time of weaning. Six of these rats were given an extra supplement of 35 μg. of riboflavin per day. At the end of 66 days the average capillary length in the control group was 0.30 mm. (S.E. = .02 mm.) and in the group receiving extra riboflavin 0.35 mm. (S.E. = .07 mm.). By the end of 131 days some of the rats in both groups had corneal vessels definitely longer than "normal". These raised the average capillary length to 0.5 mm. (S.E. = .07 mm.) in the control group and 0.64 (S.E. = .09 mm.) in the group with added riboflavin.

The failure of added riboflavin to influence the incidence of "spontaneous" capillaries in these rats as well as the proof that corneal riboflavin is not affected by ultraviolet light in spite of capillary invasion therefrom, re-emphasizes the fact that riboflavin deficiency is only one of a number of conditions which will lead to corneal vascularization.

SUMMARY AND CONCLUSIONS

1. Brilliant continuous illumination with incandescent lamps did not augment the changes in the cornea and conjunctiva resulting from riboflavin deficiency.

2. The healing of injury of the cornea resulting from ultraviolet light was handicapped by brilliant continuous illumination from incandescent lamps and by protracted riboflavin deficiency. However, the failure to observe an effect of either visible or ultraviolet light on the riboflavin content of the cornea indicates that this handicap is not the result of local destruction of riboflavin by light but due to some other cause. The healing of injury due to milder trauma, that involving primarily the epithelium, was not affected by either light or riboflavin deficiency alone under the conditions of these experiments. However, a combination of these two factors did lead to definite handicaps.

3. High riboflavin intake failed to prevent the occurrence of "spontaneous corneal vascularity" occurring in a normally nourished rat colony.

4. The variety of agents and deficiencies which will induce corneal vascularization indicates the need for caution in interpretation of corneal vascularization in man.

LITERATURE CITED

BESSEY, O. A., AND O. H. LOWRY 1944 Factors influencing the riboflavin content of the cornea. J. Biol. Chem., vol. 155, p. 635.

DEMPSEY, E. W. Personal communication.

DUKE-ELDER, SIR W. STEWART 1942 Text-Book of Ophthalmology. Vol. II, St. Louis, The C. V. Mosby Company.

LOVELAND, R. P. 1943 Simplified photomicrography with a hand camera. Science, vol. 97, p. 24.

NATIONAL RESEARCH COUNCIL 1943 Inadequate diets and nutritional deficiencies in the United States. Their prevalence and significance. Bull. National Research Council, no. 109, (Nov.).

NUTRITION REVIEWS 1943 Specificity of the ariboflavinosis syndrome in man, vol. 1, p. 327.

SYDENSTRICKER, V. P., W. H. SEBRELL, H. M. CLECKLEY AND H. D. KRUSE 1940 The ocular manifestations of ariboflavinosis. J. Am. Med. Assn., vol. 114, p. 2437.

TISDALL, F. F., J. F. MCCREARY AND H. PEARCE 1943 The effect of riboflavin on corneal vascularization and symptoms of eye fatigue in R.C.A.F. personnel. Canadian Med. Assn. J., vol. 49, p. 5.

WIEHL, DOROTHY G., AND H. D. KRUSE 1941 Medical evaluation of nutritional status. V. Prevalence of deficiency diseases in their subclinical stage. Milbank Quart., vol. 19, p. 241.

WOLBACH, S. B., AND O. A. BESSEY 1942 Tissue changes in vitamin deficiencies. Physiol. Rev., vol. 22, p. 233.

COMPARATIVE EFFECTIVENESS OF VARIOUS IRON COMPOUNDS IN PROMOTING IRON RETENTION AND HEMOGLOBIN REGENERATION BY ANEMIC RATS

SMITH FREEMAN AND MARIE W. BURRILL

WITH THE TECHNICAL ASSISTANCE OF MARGARET GRIESSER

Department of Physiology, Northwestern University Medical School, Chicago

(Received for publication June 15, 1945)

Iron compounds used for the enrichment of bread and flour should possess two qualifications. First they should be utilized by the body and secondly, they should have no adverse effect upon the preservation of flour (Fed. Reg., '41). Most compounds of iron that are readily soluble in water and dilute acid cause flour to become rancid or to have a decreased vitamin content (Gillet, '45). Sodium iron pyrophosphate seems to have no adverse effect on flour, but there is some difference of opinion as to the effectiveness of its utilization by the body. Nakamura and Mitchell ('43) reported a relatively high degree of utilization of this compound in anemic rats while Street ('43) found it to have only about half the effectiveness of ferrous sulphate in promoting hemoglobin regeneration in anemic rats.

The present study was undertaken to compare various iron compounds used for the enrichment of bread and flour as to their effect on iron retention and hemoglobin regeneration in anemic rats. The compounds were not only tested as such, but were also compared when used as the fortifying ingredient in specially prepared breads. Some observations were also made on the efficacy of two iron compounds in preventing anemia in milk-fed rats.

METHODS

Hemoglobin was determined by the method of Wu ('22) as adapted to a photoelectric colorimeter. The white blood cell pipettes used for hemoglobin estimations were calibrated on a standard blood of known iron content. The iron analyses by the thiocyanate method were done as previously described (Freeman and Ivy, '42) with only one modification. Immediately after extraction of the iron thiocyanate with iso-

amyl alcohol, the colorimeter tubes containing the extract were warmed in a water bath at 40° C. for 5 minutes and then read.

The rats used in the experiment were distributed among the various groups according to weight and sex so as to make the groups as uniform and comparable as possible.

Anemia was produced according to the method of Elvehjem and Kemmerer ('31) using cages that were described previously (Freeman and Ivy, '42). The rats were depleted on a milk diet until the hemoglobin concentration was approximately 3.0 gm./100 ml. of blood. This degree of depletion usually required 35 to 45 days after weaning (21 days of age).

The depleted rats received the iron compounds under study as a supplement to the milk diet which was offered ad libitum. The iron compounds were mixed with cane sugar in such proportion that 1 gm. of the mixture contained 0.25 mg. of iron. The iron content of the mixture was verified by analysis. That amount of iron-sugar mixture providing 0.25 mg. of iron daily for 28 days (as determined by analysis) was divided into 28 capsules (no. 000). The contents of one capsule were fed daily in a clean salt cellar with added thiamine chloride (10 γ) and copper and manganese as sulphates (0.05 mg. of each). The daily supply of milk was withheld until the supplement was consumed.

The breads containing the various iron compounds were made from a dough of the following composition:[1] flour (unenriched) 100; water 65; yeast 2; salt 2; sugar 5; milk (dried skim) 3; yeast food 0.5; and lard 2.

To this basic mixture was added an amount of iron which would give 0.20 mg. of extra iron to each 5 gm. of air-dried bread or 18 mg. of extra iron per loaf of bread.[2] The iron salt was thoroughly mixed with the dry ingredients before the dough was prepared. The baked bread was sliced, air-dried, ground, mixed and analyzed. According to the analyses, the amount of dried ground bread which contained 0.27 mg. of iron was 4.5 to 5.0 gm. This amount of bread was fed daily to the experimental animals. The control rats received the same amount of the plain bread which contained 0.07 mg. of iron by analysis. Milk was offered to the animals only after the bread was completely consumed.

The rats were killed after 28 days on the supplemented diet. Hemoglobin determinations were made on the seventh, seventeenth and twenty-eighth days.

[1] Prepared by the American Institute of Baking.

[2] This amount of iron is slightly in excess of that recommended for the enrichment of bread and flour by the National Research Council. See Bull. Nat. Res. Council, no. 110, Nov. 1944, ''Enrichment of Flour and Bread''.

In the part of the study concerning prevention of anemia in milk-fed rats by iron compounds, the weanling rats (21 days old) were given 0.25 mg. of iron daily either as ferric chloride or sodium iron pyrophosphate in a sugar mixture similar to that described above. The control group received the sugar alone. Each day, after the sugar or sugar mixture was entirely consumed, the rats were given milk ad libitum. Hemoglobin values were determined at 15, 30 and 40 days, At 40 days the rats were killed and the carcasses analyzed for iron content. Hemoglobin and iron determinations were also made on 21-day-old rats that only had access to milk since the twelfth day of life.

RESULTS

The iron content of the carcass after 40 days of depletion was found to average 0.94 mg. per rat (see table 2). This value was taken as the iron content of all the depleted rats used in this study and the retention of iron from any supplement was calculated by subtracting 0.94 from the final iron content of the carcass. The total iron intake from bread or supplement divided into the iron retained by rats on the supplement times 100 gives the percentage retention from various sources. The relative iron retention was calculated by comparing retention from other sources with that from ferric chloride. The high relative retention of the iron contained in plain bread is in accord with the finding of Smith and Otis ('37), who showed that small amounts of iron result in a relatively greater hemoglobin regeneration by anemic rats. According to these same authors, the total daily amount of iron fed in these experiments is at the upper limits of the range over which there is a direct relation between hemoglobin regeneration and iron intake. The data reported here demonstrate a direct relation between iron retention and hemoglobin regeneration.

Iron retention and hemoglobin regeneration by anemic rats on the various iron salts when fed as such or contained in bread are presented in table 1. These data show a good correlation between hemoglobin concentration and the iron content of the carcass.

The rats which received ferric chloride with sugar or in bread showed the greatest iron retention and hemoglobin formation.

Iron retention and hemoglobin regeneration for sodium ferric orthophosphate, both as the salt and in bread, and for ferric orthophosphate in bread were only slightly less than for ferric chloride iron. Reduced iron was somewhat less effective, both as the salt and in bread. Sodium iron pyrophosphate was the least effective of the compounds studied. Doubling the daily intake of this salt was without significant effect upon iron retention or hemoglobin regeneration.

TABLE 1

Iron retention and hemoglobin formation by anemic rats receiving iron supplements or iron enriched bread.

OUP NO.	SOURCE OF Fe[1]	NO. RATS IN GROUP	AVE. PERIOD OF DEPLETION	AVE. WT. AT START OF SUPPLEMENT	AVE. WT. GAIN ON SUPPLEMENT	AVE. Hb AT START OF SUPPLEMENT		AVE. Hb INCREASE ON SUPPLEMENT			AVE. TOTAL Fe CONTENT OF CARCASS			RELATIVE Hb REGENERATION IN %	RELATIVE RETENTION OF Fe	% Fe SUPPLEMENT RETAINED
			days	*gm.*	*gm.*	*gm./100 ml.*		*gm./100 ml.*		C_1[2]	*mg.*		C_1[2]		%	
1	Ferric chloride	10	34	83	102	3.07	3.01	10.65	10.00 ± 1.17		5.83	5.75 ± 0.70		100	100	68.0
2	Ferric chloride	10	36	89	107	2.96		9.72			5.60					
3	Ferric chloride	9	38	93	91	3.00		9.47			5.82					
4	Sodium iron pyrophosphate	10	35	85	92	3.04		4.76 ± 1.74		2.5	3.19 ± 0.42		3.7	48.0	46.6	32.1
5	"Double amount" sodium iron pyrophosphate	6	36	90	55	2.97		6.18 ± 1.50		2.5	2.91 ± 0.13		3.8	62.3	40.9	14.0
6	Reduced iron	10	34	84	107	2.92		8.85 ± 0.89		0.8	5.15 ± 0.59		0.6	83.4	87.5	59.3
7	Sodium ferric orthophosphate	10	39	94	88	2.97		9 26 ± 1.52		0.4	5.26 ± 0.59		0.5	93.5	89.9	61.0
8	$FeCl_3$ bread	10	39	94	86	2.92		9.28 ± 1.10			5.61 ± 0.62			100	100	61.0
9	Sodium iron pyrophosphate bread	10	34	91	83	3.00		4.09 ± 1.10		3.5	3.32 ± 0.69		2.7	44.5	51.0	30.8
10	Reduced iron bread	9	44	91	93	2.78		7.95 ± 1.64		0.7	4.86 ± 1.00		0.5	86 0	84.0	51.0
11	Ferric orthophosphate bread	9	42	92	107	3.06		8.30 ± 1.48		0.5	5.27 ± 0.74		0.4	90.5	92.7	56.5
12	Sodium ferric orthophosphate bread	9	45	94	98	2.86		8.66 ± 1.13		0.5	5.29 ± 0.73		0.3	93.2	93.1	56.7
13	Plain bread	11	40	92	69	2.92		2.38± 1.09		4.9	2.33 ± 0.36		6.4	25.9	29.8	68.0

[1] Source of iron compounds (1, 2, 3, 8) Mallinckrodt, (4, 5, 9, 12) Victor Chemical, (6, 10, 11) Merck & Co.

[2] C_1 = C.R. = critical ratio, considered significant when greater than 2 according to Fischer's rule.

There is good agreement between the results obtained when the iron salts were fed mixed with sugar and when contained in bread. The relative retention of iron from various sources is in the same order in either case. The absolute amounts retained from any given compound are so similar for iron contained in bread as compared to that mixed with sugar as to indicate that the absorption of iron was not significantly altered by its inclusion in the bread. Widdowson and McCance ('42) found that iron was absorbed by human subjects from a diet that contained 40–50% of its calories as white bread but that its absorption was reduced when white flour was replaced by one containing considerable quantities of bran.

TABLE 2

Prevention of anemia in milk-fed rats.

SUPPLEMENT	NO. RATS IN GROUP	TIME ON EXPERIMENT	INITIAL WT.	AVE. WT. GAIN ON SUPPLEMENT	AVE. Hb AT 15 DAYS OF SUPPLEMENT	AVERAGE FINAL Hb	AVE. Fe CONTENT OF CARCASS	Fe RETAINED [2]	RELATIVE RETENTION OF Fe	RELATIVE INCREASE IN HEMOGLOBIN
		days	*gm.*		*gm./100 ml.*	*gm./100 ml.*	*mg.*	*mg.*	%	%
$FeCl_3$ + sugar	7	40	30	62	13.60	13.08 ± 2.03	4.00 ± 0.97	3.06	100	100
Pyro [1] + sugar	7	40	28	80	7.87	7.20 ± 0.66	2.32 ± 0.3	1.38	45	41.5
Sugar	6	40	28	44	6.12	3.04 ± 0.72	0.943 ± 0.21			
21-day-old rats	12	0	35			9.40 ± 0.33	1.06 ± 0.14			

[1] Sodium iron pyrophosphate.

[2] Final Fe content of Fe supplemented group minus Fe content of group fed sugar alone.

A lower iron retention and hemoglobin regeneration by anemic rats fed sodium iron pyrophosphate was demonstrated in three separate experiments; first, with the compound fed at two concentrations, second, when added to bread and third when it failed to prevent the development of an anemia in rats (table 2). It does provide sufficient iron to permit growth but the retention of iron from this source was only approximately half that of ferric chloride, whether used in the treatment or prevention of anemia.

The various iron compounds show the following order of effectiveness in their relative retention by anemic rats: ferric chloride > sodium

ferric orthophosphate = ferric orthophosphate > reduced iron > sodium iron pyrophosphate (table 1). The order is the same whether the rats were fed the compounds themselves or received bread containing them. The relative degrees of hemoglobin regeneration for the compounds or enriched breads also give the same order whether determined after 7, 17 or 28 days. For this reason only the final hemoglobin values are included in the table.

Street ('43) studied hemoglobin regeneration in anemic rats and obtained results which indicate essentially the same relative utilization of ferrous sulfate and sodium iron pyrophosphate as that which we have obtained for ferric chloride and sodium iron pyrophosphate. The higher hemoglobin concentration on sodium iron pyrophosphate reported by Nakamura and Mitchell ('43) may be due to the relatively low weight gain of their rats during the experimental period. The degree of anemia was also less in their animals at the beginning of the experimental period. Iron retention was greater in 21 days for three sodium iron pyrophosphate rats reported by Nakamura and Mitchell than for our 28-day animals maintained on the same supplement, while ferric chloride retention was relatively greater in our animals.

The uniformity of iron retention and hemoglobin regeneration by anemic rats on a given compound is illustrated by the data obtained on three different groups of rats fed ferric chloride. These three groups were controls for rats fed sodium iron pyrophosphate, reduced iron and sodium ferric orthophosphate. The three groups were studied at different times and the rats were from different litters. There is good agreement among the three groups both as to iron retention and hemoglobin regeneration. Variation in hemoglobin regeneration is greater than for iron retention but the hemoglobin increase is also greater so that the impression derived from either determination is generally much the same. In studying the efficacy of a given iron compound as a source of iron, iron content appears to be a more direct measure than hemoglobin concentration. Other factors than iron absorption may affect hemoglobin regeneration and the concentration of hemoglobin in the blood is subjected to other factors that influence blood volume. The influence of growth on the concentration of hemoglobin in animals with a similar iron retention is well illustrated by groups 4 and 5, fed different amounts of sodium iron pyrophosphate (table 1). Although iron retention was essentially the same for the two groups, the hemoglobin increase of group 5 was on an average about 2 gm. higher while the weight gain of this group was only slightly more than half that of group 4.

In the prevention of anemia in weanling rats, the relative iron retention of ferric chloride and sodium iron pyrophosphate was similar to that obtained in the depleted rats (see table 2). So far as hemoglobin formation is concerned, this experiment is theoretically complicated by the fact that the diet is deficient in copper and manganese. If these two substances are supplied the development of anemia may be retarded, while if these substances are not supplied hemoglobin formation may be influenced by their absence as well as by the availability of iron. At the end of this experiment the variability both of hemoglobin and total iron content was greater for the rats fed ferric chloride than was the case at the end of the depletion experiments (see table 1).

The prevention of anemia in milk-fed rats offers a method of evaluating iron compounds which has certain desirable aspects. This procedure saves time since the experiment is ended by the time the control animals are depleted which is actually the starting point in the depletion experiments. Thus the prevention method covers a span of 40 days' time while the depletion method in our experience takes 60 to 70 days. During depletion some rats do not grow sufficiently or develop respiratory tract infections and have to be discarded as unfit for experimental material. This loss, representing considerable wasted time and effort, is reduced when the source of iron is fed from the time of weaning, only the control group being subjected to these hazards. The hemoglobin and total iron content of the twelve rats killed at weaning in the present experiment (table 2) indicate that the rats at this age were quite uniform when treated as described by Elvehjem and Kemmerer ('31).

SUMMARY

1. The retention of iron from different sources by anemic rats was qualitatively and quantitatively similar irrespective of whether the iron salts were fed as such or contained in bread.

2. The various iron compounds tested showed the following order of effectiveness with respect to the relative degree of iron retention and hemoglobin regeneration produced in anemic rats: ferric chloride > sodium ferric orthophosphate = ferric phosphate > reduced iron > sodium iron pyrophosphate.

3. Prevention of anemia in milk-fed rats given supplements of ferric chloride or sodium iron pyrophasphate for 40 days after weaning (21 days) gave results for relative iron retention and hemoglobin regeneration similar to those obtained with depleted rats fed the same supplements for 28 days.

ACKNOWLEDGMENT

We are indebted to the Victor Chemical Company for a grant which made this work possible. The authors wish to acknowledge their indebtedness also to the American Institute of Baking and to its Director, Dr. F. C. Bing, for their cooperation in this study.

LITERATURE CITED

ELVEHJEM, C. A., AND A. R. KEMMERER 1931 An improved technique for the production of nutritional anemia in rats. J. Biol. Chem., vol. 93, p. 189.

FEDERAL REGISTER May 27, 1941, vol. 6, p. 2574.

FREEMAN, S., AND A. C. IVY 1942 The influence of antacids upon iron retention by the anemic rat. Am. J. Physiol., vol. 137, p. 706.

GILLET, J. M. 1945 (Victor Chemical Works.) Personal communication.

NAKAMURA, F. I., AND H. H. MITCHELL 1943 The utilization for hemoglobin regeneration of the iron in salts used in the enrichment of flour and bread. J. Nutrition, vol. 25, p. 39.

SMITH, M. C., AND L. OTIS 1937 Hemoglobin regeneration in anemic rats in relation to iron intake, with suggestions for improvement of the bioassay technique for measuring available iron. J. Nutrition, vol. 13, p. 573.

STREET, H. R. 1943 A study of the availability of the iron in enriched bread. J. Nutrition, vol. 26, p. 187.

WIDDOWSON, E. M., AND R. A. MCCANCE 1942 Iron exchange of adults on white and brown bread diets. Lancet, vol. I, p. 588.

WU, H. 1922 Studies on hemoglobin. I. The advantage of alkaline solutions for colorimetric determination of hemoglobin. J. Biochem. (Japan), vol. 2, p. 173.

NUTRITIONAL EFFECTS ON THE GASTRIC MUCOSA OF THE RAT

I. LESIONS OF THE ANTRUM

THEODORE F. ZUCKER, BENJAMIN N. BERG AND LOIS M. ZUCKER
Department of Pathology, Columbia University, New York

FIVE FIGURES

(Received for publication May 5, 1945)

More than fifty reports can be cited in the literature of the last two decades which deal with gastric lesions of nutritional origin. Fridericia et al. ('40) and Sharpless ('40; '43) have reviewed the literature from different points of view. Attempts to establish specific deficiencies as responsible causes have not led to very acceptable conclusions. An element of complexity is introduced by the fact that three types of lesions have been identified (Berg, '42), each characteristic of one of the three types of epithelial coats which make up the gastric mucosa of the rat (figs. 1, 2 and 4) of this and figs. 1–3 of the succeeding paper). It appears that less progress has been made in clarifying the relations of individual factors to gastric epithelium than has occurred in the more recent field of study which deals with skin epithelium and the factors necessary for its normal maintenance.

Orienting experiments were begun several years ago with repetitions of the experiments of other investigators. While our first observations were based on frankly multiple deficiency diets (e.g., diet 872 of table 2) on which all three types of lesions can be produced in the same rat, our aim was to identify individual deficiencies with the abnormal behavior of the epithelium. We have published two brief reports on antral lesions and calcium deficiency (Zucker and Berg, '43; '44). This and the following paper deal with more detailed observations on all three regions of the stomach.

TECHNIQUE

To prepare a stomach for observation the esophagus is cut at its entrance to the stomach and about 1 cm. of duodenum is left attached. The stomach is then cut open along the greater curvature and stretched out with pins on cardboard prior to fixation in 4% formaldehyde solu-

tion (a 1 to 10 dilution of "formalin"). This places the region of the lesser curvature (called antrum or prepyloric region) in the center with the two parts of the cut fundus (also called body or fornix) on the sides and the rumen (forestomach) above. The esophageal opening is in the center of such a stretched specimen. Figure 1 shows a normal rat's stomach photographed against a white background to show the

Fig. 1 Stomach of a normal rat opened along the greater curvature and stretched out flat on cardboard. This stomach was photographed against a white background and in the fresh state thus retaining the color in order to differentiate the various regions. All the other specimens in this and the succeeding paper were fixed in formalin and have lost the natural color of the fundus. They were photographed against a black background so that the lesions would show to better advantage. The transparent area above the dividing ridge is the rumen (forestomach). Below the ridge is the glandular part of the stomach. Separated from this by the pylorus is the duodenum. The glandular area is subdivided into the fundus (dark, thicker) and the antrum (translucent, thinner). The esophagus enters near the juncture of the three regions. In the rat the antrum secretes no acid whatsoever — only mucus. The glands of the fundus contain the acid and pepsin secreting cells. This region is also the most vascular.

transparent nonsecretory rumen above the dividing ridge which separates it from the glandular portion consisting of the acid secreting fundus and the non-acid mucus secreting antrum. The latter portion of the stomach has a smooth translucent appearance. In the normal stomach the only interruption of the smooth surface consists of lymph nodules which can be distinguished from lesions in microscopic section and which when counted in groups of animals average one or less per rat.

The appearance of the typical antral lesions of calcium deficiency is shown in figure 2. The circumscribed raised areas are due to hyperplasia of the glandular epithelium. The center usually shows a pin-

Fig. 2 Antral lesions (4-week Ca deficiency). Rumen and fundus are normal. Lesions in the antrum are sharply circumscribed raised areas with central pits or grooves which may show either a white or red central dot or may be clear. The location of most of the lesions near the pylorus, as in this figure, is rather typical although at times the whole antral area may be covered.

point hemorrhage or a white plug consisting of fibrin and leucocytes with hemoglobin no longer present. The variation in size of the individual lesions in a given stomach is not always as large as shown in figure 2. Without microscopic examination of sections a lymph nodule cannot always be distinguished from a hyperplastic lesion. In the tables the severity of the condition is expressed by the incidence figure (i.e., fraction of animals showing abnormal antrum) and a mean count of lesions per rat.[1]

TABLE 1

Production of lesions by calcium deficiency.

SERIES [1]		ADDED		WEIGHT [2] IN GM. AT		NO. OF RATS	ANTRUM	
		Ca	P	28 days	56 days		Count	Incidence [3]
1	Control	0.6	0.4	49	105	124	0.5	4%
	Experimental	0.0	0.4	53	77	134	11.9	88%
2	Control	0.6	0.4	65	174	94	0.2	2%
	Experimental	0.0	0.4	65	101	141	13.6	85%

[1] Series 1 has 20% protein, series 2 has 27%. Series 2 has more B complex than series 1. For composition of diets see table 2; diets 904, 905 and 1009 are typical members of series 1 and diets 1015–1017 and 1029 are typical members of series 2.

[2] Sexes are equally distributed in each group. There is always a large sex difference in growth in the control diets of series 2, less difference in the control diets of series 1 (due to protein content which is suboptimal for males, Zucker and Zucker, '43; '44) and little sex difference on the Ca deficient diets. The rats in series 2 were larger throughout. On the same stock diet within the same rat strain the breeders had been selected from large sized parents for several generations.

[3] See footnote 1 below.

EFFECT OF CALCIUM DEFICIENCY

Table 1 presents two series of experiments, comprising a total of 493 rats. In each series the salt mixture of the control diets contributes 0.6% Ca, while in the experimental diets calcium addition was omitted. Nearly 90% of the experimental animals showed a definitely abnormal antrum, while the control diets resulted in a normal gastric mucosa

[1] In the incidence figure we consider an antrum not normal when more than one raised area is seen. This introduces less error than the inclusion of normal lymph nodules. The mean count includes the lymph nodules and is of interest only if well removed from unity. Statistical comparisons of groups can be based on either kind of observation, using "t" for mean count and chi square for incidence. The mean count appears to be by far the more precise measure. We give the incidence also because it has been the more customary measure and is the only one which applies in studies of rumen and fundus.

which did not differ from that in the 198 animals of corresponding age that were fed the stock diet. Included among the controls of the second series are forty-eight rats on diets in which the B factors were supplied in the form of liberal allowances of thiamine, riboflavin, pyridoxine, pantothenic acid and in some cases choline. As stated elsewhere on the basis of a smaller series (Zucker and Berg, '43), the experimental animals of series 2 showed lesions exclusively in the antrum, while those of series 1 also had defects in other parts of the gastric mucosa. The nature and incidence of the latter are discussed elsewhere (Zucker, Berg and Zucker, '45).

TABLE 2

Composition of diets.[1]

DIET	PROTEIN	DIETARY Ca	DIETARY P	B COMPLEX SUPPLIED	CARBO-HYDRATE
		%	%		
872	12% Labco casein	0.80	0.34	....	starch
896	20% Labco casein	0.60	0.41	....	sucrose
904	20% Egg albumen	0.61	0.45	5% Vitab	sucrose
905	20% Egg albumen	0.010	0.45	5% Vitab	sucrose
909	20% Egg albumen	0.012	0.48	10% Vitab, 1.5 mg. B_2	sucrose
913	20% Egg albumen	0.010	0.044	5% Vitab	sucrose
1009	20% Fibrin	0.044	0.49	10% Vitab, 1.5 mg. B_2	sucrose
1010	20% Fibrin	0.64	0.43	....	sucrose
1015	27% Labco casein	0.60	0.41	5% Vitab, 0.1 mg. B_2, 0.5% yeast extract	cerelose
1016	27% Labco casein	0.0037	0.41	5% Vitab, 0.1 mg. B_2, 0.5% yeast extract	cerelose
1017	27% Labco casein	0.0078	0.46	5% Vitab, 0.1 mg. B_2, 2.0% yeast extract	cerelose
1019	27% Labco casein	0.60	0.40	....	starch
1029	27% Labco casein	0.017	0.41	5% yeast extract	cerelose
1035	27% Labco casein	0.24	1.24	5% Vitab, 0.25 mg. B_2, 0.75 mg. Ca pantothenate	cerelose

[1] All diets in this and the following paper have a total of 2% added fat, in the form of refined cottonseed oil containing 750 units vitamin A activity as carotene. Diets 872, 896, 1010 and 1019 contain 0.3% cod liver oil (replacing an equal weight of cottonseed oil). Diets 872 and 896 have 1% NaCl. All other diets contain 1.2% modified (Ca, P free) Wesson salt mixture. Ca is added as $CaCO_3$, P as KH_2PO_4. The calculated Ca and P contents include the contributions from all dietary constituents. Our analyses indicate the following Ca/P contents for the materials as used: Labco casein 0.003/0.79; hot ethanol extracted fibrin 0.2/0.11; heated egg albumen 0.04/0.084; yeast extract (Standard Brands brewers' yeast extract type 3) 0.27/3.5; Vitab (rice bran extract, Vitab type 2) 0.034/0.54.

In line with the extensive studies of Sherman and of Steenbock, 0.6% Ca has been found to be a reasonable value for normal control diets. We have only roughly explored the region between near zero content of Ca and this normal, or the effect of varying the Ca/P ratio of the diet on the antral count. In the case of studies on bone (Shohl, '36) or

on parathyroid (Stoerk and Carnes, '45) significant observations are possible over a wide range of Ca and P because both Ca and P deficiency play a role. Since even extreme P deficiency[2] does not lead to antral lesions, the Ca/P ratio is of significance only insofar as it limits the absorption of Ca which is, in this sense, specific. Table 3 shows some available data, of which the most striking feature is perhaps the persistence of the lesions up to a Ca content of 0.2%. Figure 3 is a dose-action curve

TABLE 3

Effect of Ca level (%) and Ca/P ratio on incidence and lesion count.[1]

Ca %	0.010	0.01	0.017	0.2	0.26	0.24	0.41
P %	0.044	0.43	1.89	0.24	0.42	1.24	0.45
Ca/P	0.23	0.023	0.009	0.83	0.62	0.19	0.91
Count	5.0	12.7	21.1	2.8	6.3	4.4	0.4
Incidence	18/29	238/275	11/11	20/51	21/29	4/8	0/22

[1] All of the experiments are of 4 weeks' duration and the diets contain 20 or 27% protein, adequate B complex and no vitamin D. Since the data do not represent matched animals or even contemporary experiments, and since the numbers in some groups are quite small, no precise comparison is intended. However, the simple relation between Ca content of the diet (when P is kept constant) and lesion count displayed in figure 3 indicates that the results are at least fairly consistent throughout. The lesions disappear at 0.4% Ca and are still present at around 0.2% Ca. There is a distinct effect of the Ca/P ratio which seems most marked at the lowest Ca level.

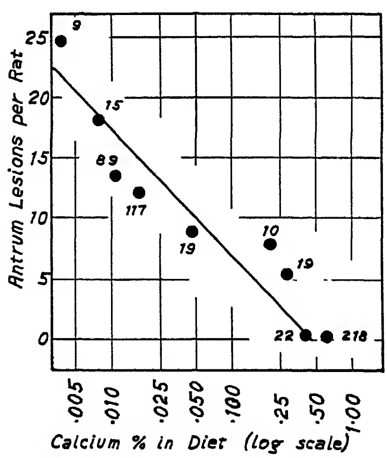


Fig. 3 Conventional dose-action curve (action = antrum count plotted against log dose = log % Ca in the diet). Numbers indicate the number of rats averaged for each point. All the diets contain 0.4 to 0.5% P and all experiments were run for 4 weeks. Differences in Ca content result from difference in added $CaCO_3$, as well as varying contributions from the other components of the diet. The data are taken from tables 3 and 6.

[2] We need not detail the results with current rickets producing diets used in vitamin D testing as well as several diets low in phosphorus and otherwise adequate, since they were uniformly negative.

for all the data on diets in which the Ca varies from 0.0037 to 0.6% and the P is kept constant between 0.4 and 0.5% : the relation between antral count and Ca content of the diet under these conditions is good enough considering the fact that the animals were not matched and the data were collected over a period of 4 years.

TABLE 4

The effect of vitamin D on lesions of the antrum.

DIET	CA%	P%	CA/P	WEIGHT (GM.) 28 days	WEIGHT (GM.) 56 days	ANTRUM Count	ANTRUM Incidence[1]	σ[2]	t	t 5% / 1%
913				49	81	4.6	15/23	4.72		2.0
	0.010	0.044	0.23						2.3	
913 + D				49	84	1.6	6/17	2.59		2.7
905, 909				49	57	16.0	22/22	5.28		2.0
	0.011	0.46	0.024						5.4	
905 + D, 909 + D				50	76	5.4	13/21	7.35		2.7
1029				64	119	11.3	16/18	7.84		2.0
	0.017	0.41	0.041						2.8	
1029 + D				64	153	4.6	11/18	6.47		2.7
1035				60	130	4.4	4/8	6.32		2.7
	0.24	1.24	0.193						1.7	
1035 + D				61	163	0	0/6	0		3.0

Variance analysis of the above data.

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F	F–1%
Total	132	57.0		
Experimental error	125	34.4		
Vitamin D treatment	1	1256	37	6.8
Diet (Ca/P level, ratio)	3	491	14.3	3.9
Interaction of diet and D effects	3	165	4.8	3.9

[1] See footnote 1, p. 304.

[2] σ is the standard deviation of the antrum count. The probability ratio "t" is the measure of the significance of the difference in the means for the individual comparisons; "F" is the significance measure for the variance analysis. The values which "t" or "F" must equal or exceed for the 1% or 5% levels of significance are also tabulated. (For methods, and the meaning of "t" and "F" see Snedecor, '40.) Obviously the D effect is overwhelmingly significant, the Ca/P level and ratio have very significant effects, and the extent of the D effect depends on the Ca/P level and ratio (interaction term). The serum Ca on 1029 + D (8.1 mg. per 100 ml.) is distinctly below normal although higher than the level found for 1029 (6.2).

Each comparison (with and without D) was made at the same time on rats matched for weight, sex and litter origin.

Administration of vitamin D

If the effect on the antral mucosa is due to Ca deficiency, one might look for relief of the condition when vitamin D is fed. With extreme low calcium content of the diet complete prevention cannot be expected since not enough calcium is fed for the D action to lead to much improvement of the net calcium balance. The definite existence of a D effect as shown in table 4 is further confirmatory evidence that the pertinent deficiency is in fact calcium. Incidentally, rats produced on diets containing cod liver oil have been found to be unsatisfactory subjects for this kind of study.

Neutralization of gastric acidity

In the therapeutics of gastritis and gastric ulcers in man the administration of acid binding substances, buffers and neutralizing agents has played a large role. The underlying thought is that the pathology is referable to the irritating action of the acid. A good deal of experimental work has been aimed at ascertaining whether or not HCl can be expected to have such action on living mucosal epithelium. Henning and Norpoth ('32) after an impartial review of both sides of the problem conclude that neither in their own work nor in any previous publications is there any cogent proof for either side of the argument. In view of the fact that the sole difference between some of our experimental and control diets consists of quite appreciable amounts of $CaCO_3$, it seemed worthwhile to ascertain whether other materials having equivalent neutralizing action on the gastric contents would also act as preventives. Table 5 indicates that three agents which have been used therapeutically, namely, bicarbonates, secondary phosphates, and aluminum hydroxide, have no discernible alleviating effect on antrum lesions produced in the manner here described. The phosphate apparently makes things worse. This latter result is additional evidence for a specific calcium effect.

Possible factors concerned in the hemorrhage

Our own observations as well as those of others indicate a generalized tendency to hemorrhage in calcium deficiency (Zucker and Berg, '44). Since hemorrhage is one phase of the antral pathology, attempts were made to find some intermediate processes leading to such an end result. Deficiency of ascorbic acid and vitamin K lead to hemorrhage and both of these substances are synthesized in the normal rat. The thought that calcium might be involved in either of these syntheses seemed worth

investigating. Conditions which will disturb ascorbic acid synthesis in the rat have been reported (Vedder, '38; Feng, King and Longenecker, '44). McCollum in a discussion of calcium deficiency and hemorrhage suggests "that studies should be made of the possible relationship between calcium deficiency and ascorbic acid synthesis" (McCollum et al., '39, p. 158). The possibility of inadequate ascorbic acid production as a

TABLE 5

Effect of alkalinizing agents on antral lesions.

SUPPLEMENTS TO DIET 1029 [1]	DIETARY CA %	DIETARY P %	WEIGHT (GM.) AT 28 days	WEIGHT (GM.) AT 56 days	WEIGHT (GM.) AT 62 days	SERUM CA	ANTRUM Count	ANTRUM Incidence [2]	ANTRUM σ
... .	0.017	0.41	64	119		6.2	11.3	16/18	7.84
1.5% $CaCO_3$	0.62	0.41	64	194		12.0	0.06	0/18	0.24
0.8% $Al(OH)_3$	0.017	0.41	64	120		5.9	14.2	10/12	11.1
1.2% $NaHCO_3$ 1.6% $KHCO_3$	0.017	0.41	63	120		5.5	14.4	10/12	12.4
3.2% Na_2HPO_4 4.6% K_2HPO_4	0.017	1.89	63	98		5.4	21.1	11/11	7.67
1.5% $CaCO_3$, 6 day curative			63	118	147	11.5	2.1	5/12	3.17

[1] The bicarbonate is equivalent in acid neutralizing power to the $CaCO_3$. The effective neutralizing power per gram for gastric acidity of the $Al(OH)_3$ is not immediately obvious; statements on dose appear to be based on its action as a triacid base, and we have supplied it accordingly. The secondary phosphate was fed in relatively larger amounts because we wanted to get a maximal effect and knew from previous experiments that the animals would take this much. The effect of adding $CaCO_3$, either throughout or for a curative period of 6 days, is highly significant ($t = 6.1$ and 3.8, respectively). The effect of adding bicarbonate or $Al(OH)_3$ is not at all significant. The increase in lesion count due to secondary phosphate is highly significant ($t = 3.3$) and this can be ascribed to the much less favorable Ca/P ration. The two $CaCO_3$ experiments — preventive and curative — give results significantly different from each other ($t = 2.7$) indicating that a 6-day cure is incomplete.

[2] See footnote 1, p. 304.

factor in the stomach condition was tested by feeding to a group of rats on the calcium deficient diet 1029 a daily dose of 10 mg. of ascorbic acid (freshly dissolved in 0.1 ml. water). The group receiving the supplement of ascorbic acid averaged seventeen lesions per rat, which is a bit high when compared with contemporary controls. With regard to the occurrence of a secondary K deficiency both feeding of 10 mg. K_1 (2-methyl, 3-phytyl, 1,4-napthoquinone) per 100 gm. diet, and prothrombin estimations (Campbell et al., '41) were resorted to. The experimental and control groups, each consisting of nineteen rats, showed no differ-

ence in the intensity of the hemorrhage, and both antral counts were fifteen per rat. The prothrombin times for the control and K-fed animals averaged, respectively, 31.3 and 31.2 seconds; the difference was not significant. Supplementation with calcium did not affect the prothrombin time.

Other possible mechanisms of the calcium effect

Further investigations of possible mechanisms including tissue analysis are under way, but so far little progress has been made toward establishing a connection between calcium deficiency and either the hyperplasia or the hemorrhage. There are persistent indications in the cancer literature (for review, see Shear, '33) that hyperplasia of epithelial tissue is associated with a local low concentration of calcium. If some of the older interpretations are brought in line with more recent findings (Carrothers and Suntzeff, '44) many of the inconsistencies disappear. In tissue cultures, Simms ('44) found that growth of epithelial cells is favored by low-calcium and high-phosphate content in the medium, while the opposite is true for connective tissue. Interesting relations of calcium to mucus secreting glands (the rat's antrum is composed entirely of this type) have been studied by Grant ('42). The problem is not without interest when we consider that calcium deficiency leads to highly reproducible lesions in circumscribed areas limited to one region of one part of the gastrointestinal tract with hemorrhage in other regions showing up only much later and hyperplasia appearing in no other places.

EFFECT OF B DEFICIENCY

In a paper entitled "Incidence of gastric ulcer in albino rats fed diets deficient in vitamin B (B_1)," Dalldorf and Kellogg ('32) describe lesions [3] similar as to their location and identical in their histology with those which we have obtained in calcium deficiency. Such results were also reported by Sure and Thatcher ('33) and apparently by Drummond et al. ('38). Drummond's characterization of his ulcers is somewhat uncertain. We also have verified these findings (line 1, table 6, and fig. 4). The lesions are obtained in 4 weeks on diets supplemented with pure B factors other than thiamine, with autoclaved yeast (as in Dall-

[3] The meaning of "ulcer" and "erosion," the other term frequently used, is highly dependent on the school of thought to which the investigator belongs. In order to avoid arguments not pertinent to the experimental mucosal changes here under discussion, we use the term "lesion" concerning which there need by no argument. We have, however, no particular quarrel with anyone who wishes to call his lesions ulcers.

dorf's experiments), or with B complex deficiency. Dalldorf identified the lesions only microscopically and deduced their location from the histology of the surrounding tissue. In properly stretched specimens, the lesions can be seen grossly. They are neither as large nor as prominent as those in animals subjected to calcium deficiency for an equal length of time, nor is the incidence or the mean count ever as high. In all respects they resemble the appearance obtained after about 2 weeks on calcium deficiency. They are not produced by less than 4 weeks of thiamine deficiency.

TABLE 6

Effect of calcium level in B deficient diets and of thiamine level in calcium deficient diets.

CALCIUM	THIAMINE	NO. OF RATS	ANTRUM COUNT	σ	t
gm./100 gm.	*mg./100 gm.*				
0.6 or 0.8 [1]	no thiamine	307	5.34	5.6	
	or				7.4
1.9 to 3.8 [2]	no B complex	97	1.02	2.1	
0.01	0.75	47	12.7		
0.04		10	9.7		
0.0037	1.2	9	24.7		
0.01	1.5	42	14.5		
0.04		9	8.7		
0.0078	2.55	15	18.1		
0.017	4.5	117	12.1		

[1] Included in line 1 are all of the animals on diets 872, 896, 1010 and 1019.

[2] Included in line 2 are 47 rats on the autoclaved stock diet, plus rats on diets 872, 896, 1010 and 1019 with additional Ca and P. In all cases the Ca/P ratio is 1.5 to 2.0. The preventive effect of additional Ca in a B deficient diet is highly significant. The further addition of B complex (since thiamine is the single B factor whose absence leads to antrum lesions, the B complex addition is measured by thiamine content calculated from data supplied by the manufacturers of the various B concentrates) has no effect on the lesion count. The series of diets with B complex present all have a P content of 0.4 to 0.5%. The antrum count on these diets is quite definitely dependent on the Ca content, not the thiamine content.

Prevention with increased calcium

In the last week on a B deficient diet the food intake is reduced to roughly 15% of the intake for the same period of a calcium deficient diet and 10% of the intake of a normal diet; the calcium level in the diet under these conditions can be misleading as far as the relative calcium intake is concerned. It appears to be true (table 6) that added calcium supplements entirely prevent the lesions even if the food intake is limited by thiamine deficiency. On the other hand, it is seen in table 6 that

adding thiamine to a diet already adequate in this respect but deficient in calcium has no effect on the antral count.

The data show clearly that presence or absence of the disturbance in the mucosa is dependent on calcium. The reason, however, why thiamine deficiency should have an adverse effect which is mitigated by added

Fig. 4 Antral lesions (4-week B complex deficiency). The lesion defects are small pits accompanied by little or no surrounding hyperplasia. This stomach also illustrates the occurrence of fundic hemorrhage (see succeeding paper).

calcium cannot be established from the data now available. We do not know (1) whether in intermediary metabolism the calcium requirement is increased by thiamine deficiency, or (2) whether a mucosa deficient in both thiamine and calcium is more subject to attack (or less capable of carrying on healing processes) than a purely calcium-deficient one, or (3) whether the efficiency of calcium absorption is reduced by thia-

mine deficiency, or (4) whether the decrease in food intake lowers the calcium ingestion to the point where deficiency symptoms are produced. Attempts now under way to establish a separate, possibly partial, role for thiamine in the production or exacerbation of the lesions have not yet yielded conclusive results; neither is there cogent evidence that the thiamine deficiency operates exclusively through an effect on calcium, either in the total or the intermediary metabolism of this element.

A principal difficulty with further experimental work is the variability of results with B deficiency. With calcium deficiency about 90% of the several hundred individual animals show disturbances of the antrum in 4 weeks and every group gives an appreciable count. The lack of B, however, gives no such regularity of results. The individual high counts of over thirty lesions given by calcium deficiency are never reached, the overall mean is distinctly lower (about 5 against 12) and group means vary markedly including many zero values. We at first ascribed this to a seasonal effect, since for 2 successive years we obtained negligible counts during the summer months. However, it now appears that this phenomenon of refractoriness need not be seasonal.

Effect of food restriction

During the past winter paired feeding experiments with total B deficiency were carried out. The deficiency, or the food restriction in the control animals, was so severe a strain that a number of deaths occurred in both groups. Of the animals which survived the 4 weeks (nine pairs and ten extra food restriction controls), one animal on B deficiency and two on food restriction showed results in the antrum. We may have had a run of animals more refractory to antrum effects and more sensitive to the general B deficiency effects. The labor expended on each rat is considerable, partly because the danger of sudden death from starvation induced fundic hemorrhage (Zucker et al., '45) makes it necessary to feed the restricted animals (controls) twice every 24 hours.[4] Apparently in the face of this extreme variability it will require a major effort to solve the problem along these lines.

[4] Besides matters of rationale, the paired feeding experiment presents particular problems of technique when one deals with highly reduced food consumption. For instance, while the B deficient animals consume their reduced intake fairly continuously through the 24 hours, the artificially restricted controls, having been completely starved for 24 hours, with normal feeding urge madly pounce on their food (or even the hand which feeds them) and consume what they get in a few minutes. To make conditions more similar factually the allotted food is given to the control animals in divided portions. The difference between a single and divided restricted allotment has been pointed out by Mitchell (J. Biol. Chem., vol. 58, p. 918 (1923)).

STATISTICAL CONSIDERATIONS

Cowgill ('39) in a discussion of observations such as those of Dalldorf and Kellogg, states: "The failure to secure ulcers in practically every case . . . constitutes an argument in favor of the view that the ulcers arise secondarily in the course of vitamin B_1 deficiency, probably as part of the response of the stomach to a generalized systemic condition and not because the function of vitamin B_1 is specific in relation to this organ." Applying Cowgill's criteria to our 8 weeks' calcium deficiency experiments (Zucker and Berg, '44) would permit the conclusion

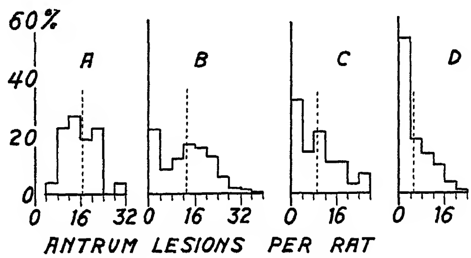


Fig. 5 The intervals on the horizontal axis represent 0–3, 4–7, etc., lesions per rat, with only the points 0, 16, and 32 numbered. The vertical axis represents the frequency in % for the various intervals on the lesion count scale. The mean in each case is indicated by the dotted line.

A: Ca deficient diet 1029 from 4 to 12 weeks of age; n = 26.

B: All Ca deficient diets series 1 and 2 from 4 to 8 weeks of age; n = 275.

C: Ca deficient diet 1029 from 4 to 6 weeks of age; n = 28.

D: B complex deficient diet 872 from 4 to 8 weeks of age; n = 234.

We have been unable to fit these curves with binomial distributions.

For the rationale of applying significance tests to data with very a-normal distribution curves (as we have done in table 6) see Treloar ('36, pp. 28, 127–128). The chief requirement for applying such tests is that the frequency distribution curve of means of a large set of groups like the ones being compared be approximately normal, and this will tend to be true even though the individual observations are quite a-normal in distribution, if the groups compared have large n values. The distribution of the means of the various experimental groups run on diet 872, with n values of 8 to 14, is slightly more normal than type B above, so groups of 97 or more should be satisfactory. A difference with a significance ratio of over 7, as in table 6, must be accepted as significant.

that the function of calcium is "specific in relation to this organ" since every one of the rats showed lesions (and this is true also of the additional animals run for 8 weeks since the appearance of last year's report). How generally this criterion can be applied is another matter. The frequency distribution graphs of figure 5 are pertinent. For four groups with diet and time variations, and having mean values of 16, 13, 9 and 5 lesions per rat, respectively, the distributions show striking differences. The similarity of the B deficient and short-time calcium

deficient groups would be in agreement with the interpretation that B deficiency is equivalent to a short-time calcium deficiency.

Distributions like D in figure 5 are held to be rare in biological data, and a distribution like B, taken by itself, would probably be interpreted as showing a mixture of two populations of inherently different susceptibility. However, taken together A, B, C, and D appear to be a series of steps representing decreasing intensity of stimulus. The least intense stimulus (D) is below the threshold for all of those more resistant individuals which spread themselves over several groups on the left side of curve A; with the sub-threshold stimulus they all pile up in the 0–3 category in graph D. No stimulus at all leads to a "distribution curve" represented by 100% in the lowest category. Presumably, if death did not intervene, a sufficiently extreme deficiency for a sufficiently long time would cause every antrum to be uniformly covered with lesions, and the distribution curve would have 100% of the rats in some limiting highest category determined by the anatomy of the rat's stomach.

We have been unable to associate the susceptibility in terms of lesion count in a 4-week experiment with anything known about the history and performance of the individual. Variance analysis of a suitable experiment involving 102 rats indicates that neither sex, initial weight nor litter origin affected the susceptibility significantly; in the case of sex there was no effect, while with initial weight and litter origin there were effects which would, if real, require many hundreds of rats to establish them as statistically significant. We nevertheless continue to match by these three criteria in setting up experiments, for whatever contribution to consistency there may be.

SUMMARY

1. Further evidence is adduced for the conclusion that in rats a rather early manifestation of calcium deficiency is found in the type of gastric lesion which occurs in the mucosa of the antrum. The changes consist of necrosis, hemorrhage and epithelial hyperplasia. Addition of phosphate accentuates the lesions, while vitamin D administration definitely reduces their number.

2. It is true, as others have found, that thiamine or total B complex deficiency will produce small lesions of the same type. However, while calcium increments in the diet abolish the lesions of thiamine deficiency, added thiamine has no preventive effect in calcium deficiency.

3. The administration of neutralizing agents as such has no effect on the lesions, indicating the improbability that gastric HCl plays a role in their formation.

ACKNOWLEDGMENT

We wish to acknowledge the assistance at various times of Mrs. Margaret Young Fitzgerald, Mrs. Lilian Hall Tully, Miss Frances Lauber, Mrs. Lilian Safina, Mrs. Thelma Stanley Stout, Miss Virginia Babcock, and Miss Patricia Hollister in the work involving this and other reports on gastric lesions.

LITERATURE CITED

BERG, B. N. 1942 Pathological changes in nutritional gastritis in rats. Am. J. Path., vol. 18, p. 49.

CAMPBELL, H. A., W. K. SMITH, W. L. ROBERTS AND K. P. LINK 1941 Studies on the hemorrhagic sweet clover disease. II. The bioassay of hemorrhagic concentrates by following the prothrombin level in the plasma of rabbit blood. J. Biol. Chem., vol. 138, p. 1.

CARROTHERS, C., AND V. SUNTZEFF 1944 The role of calcium in carcinogenesis. Science, vol. 99, p. 245.

COWGILL, G. R. 1939 Chapter VIII. The Physiology of Vitamin B_1. In The Vitamins: A Symposium. Am. Med. Assn., p. 163.

DALLDORF, G., AND M. KELLOGG 1932 Incidence of gastric ulcer in albino rats fed diets deficient in vitamin B (B_1). J. Exp. Med., vol. 56, p. 391.

DRUMMOND, J. C., A. Z. BAKER, M. D. WRIGHT, P. M. MARRIAN AND E. M. SINGER 1938 The effects of life-long subsistence on diets providing suboptimal amounts of the "vitamin B complex." J. Hygiene, vol. 38, p. 356.

FENG, K. C., C. G. KING AND H. E. LONGENECKER 1944 The relation of thiamin to ascorbic acid synthesis. Abstr. 107th meeting Am. Chem. Soc., p. 13B.

FRIDERICIA, L. S., S. GUDJONSSON, B. VIMTRUP, S. CLEMMESEN AND J. CLEMMESEN 1940 Stomach lesions in rats kept on diets deficient in vitamin A. Am. J. Cancer, vol. 39, p. 61.

GRANT, R. 1942 Calcium in gastric mucus and regulation of gastric acidity. Am. J. Physiol., vol. 135, p. 496.

HENNING, N., AND L. NORPOTH 1932 Untersuchungen über den Selbstschutz des Magens gegen Salzsäureverätzung. Arch. f. exp. Path. u. Pharm., Bd. 167, S. 224.

McCOLLUM, E. V., E. ORENT-KEILES AND H. G. DAY 1939 The Newer Knowledge of Nutrition. Ed. 5, p. 158.

SHARPLESS, G. R. 1940 Prevention of hyperplasia in the forestomach epithelium of rats fed white flour. J. Nutrition, vol. 19, p. 31.

——— 1943 Diet and epithelial hyperplasia in the forestomach of rats and mice. Cancer Res., vol. 3, p. 108.

SHEAR, M. J. 1933 The role of sodium, potassium, calcium and magnesium in cancer: A review. Am. J. Cancer, vol. 18, p. 924.

SHOHL, A. T. 1936 Rickets in rats. XV. The effect of low calcium — high phosphorus diets at various levels and ratios upon the production of rickets and tetany. J. Nutrition, vol. 11, p. 275.

SIMMS, H. S. 1944 Personal communication.

SNEDECOR, G. W. 1940 Statistical Methods. Iowa State College Press.

STOERK, H. C., AND W. H. CARNES 1945 The relation of the dietary Ca: P ratio to serum Ca and to parathyroid volume. J. Nutrition, vol. 29, p. 43.

SURE, B., AND H. S. THATCHER 1933 Avitaminosis. XVI. Production of gastric ulcers in the albino rat as a result of specific influence of deficiency of vitamin B. Arch. Path., vol. 16, p. 809.

TRELOAR, A. E. 1936 An Outline of Biometric Analysis. Burgess Publishing Co.

VEDDER, E. B., AND C. ROSENBERG 1938 Concerning the toxicity of vitamin A. J. Nutrition, vol. 16, p. 57.

ZUCKER, T. F., AND B. N. BERG 1943 Calcium deficiency and gastric lesions in the rat. Proc. Soc. Exp. Biol. and Med., vol. 53, p. 34.

——— 1944 The time factor in the production of gastric lesions on low calcium diets. Proc. Soc. Exp. Biol. and Med., vol. 57, p. 1.

ZUCKER, T. F., AND L. M. ZUCKER 1943 The protein requirement for growth in male and female rats. Abstr. 105th meeting Am. Chem. Soc., p. 5B.

——— 1944 Significance of the protein level in synthetic diets. Proc. Soc. Exp. Biol. Med., vol. 55, p. 136.

ZUCKER, T. F., B. N. BERG AND L. M. ZUCKER 1945 Nutritional effects on the gastric mucosa of the rat. II. Lesions of the fundus and rumen. J. Nutrition, vol. 30, p. 319.

NUTRITIONAL EFFECTS ON THE GASTRIC MUCOSA OF THE RAT

II. LESIONS OF THE FUNDUS AND RUMEN [1]

THEODORE F. ZUCKER, BENJAMIN N. BERG AND LOIS M. ZUCKER
Department of Pathology, Columbia University, New York

THREE FIGURES

(Received for publication May 5, 1945)

In the preceding paper (Zucker et al., '45) we have dealt with nutritional effects on the gastric antrum. Our original interest was principally centered on this phase of the work, the antrum being the region of the stomach most severely affected by spontaneous lesions of both man and animals. We have, however, fully recorded all the lesions produced by the rations fed; over 3000 stomachs have been examined. While a rumen is found in ruminants [2] and among laboratory animals only in the rat, mouse and hamster, the extreme hyperplasia which can be caused in the rumen of the rat by a variety of deficient diets has attracted the attention especially of cancer students. As a matter of fact the vast majority of studies involving dietary effects on the gastric epithelium have dealt with the rumen rather than the glandular portion of the stomach. The appearance of fundus and rumen lesions is illustrated in figures 1, 2 and 3.

GENERAL DISTRIBUTION OF MUCOSAL DEFECTS

The distribution of mucosal defects produced by six different rations [3] which were fed to larger groups of rats is not without interest. As can

[1] Part of this paper was reported at the 107th Am. Chem. Soc. meeting, Cleveland, 1944.

[2] The common spontaneously occurring gastric ulcers of ruminants are found not in the rumen but in the antrum. In Switzerland and Germany considerable attention has been given to this subject (for general discussion see Bongert, '12; many interesting practical details have been recorded by Tantz, '12; the pathology has been worked out by Konjetzny and Puhl, '26). Konjetzny states that during the months of February, March and April any slaughter house will afford ample material. The lesions are found in calves and young cattle which have been weaned at an early age and kept on various rations of haphazard composition. The condition has been described in the United States, but is apparently of little interest since the use of special calf meals and gruels is quite general. As to the cause the only suggestion made so far has been that of mechanical injury (straw, etc.) in spite of the fact that there is general agreement that mere trauma of the mucosa promptly heals and does not lead to ulcers. Nutritional factors obviously cannot be neglected, and, among other things, the markedly seasonal incidence suggests the involvement of calcium in analogy to the rat data presented in the preceding paper.

[3] The diets are given in table 2 of the preceding paper (Zucker, Berg and Zucker, '45).

be seen in table 1, line 1, on diet 872 which is deficient in protein and is low in B complex factors, rats will develop all three types of lesions. Of the 234 animals examined, 55% had lesions of the rumen while the fundus of 13% showed the typical hemorrhages. Antral lesions were present in 67%, with a mean count of 4.9 per rat. Lines 2 and 3 in the table refer to diets from which the calcium of the salt mixture has been

Fig. 1 The fundus is spotted with large and small adherent blood clots overlying necrotic areas, some of them arranged in streaks. The antrum and rumen are free of lesions. This is an example of severe fundic involvement. Figure 4 in the preceding paper (Zucker et al., '45) illustrates moderate involvement. The mildest form of this lesion is seen in a few pin-point hemorrhages. As against the discrete and regular appearance of the antral lesions it will be noted that the lesions of the fundus and rumen (see also figs. 2 and 3) are irregular in size and shape and thus do not permit any satisfactory counting procedure.

omitted (series 1 and 2 of the preceding paper). The first of these contains 20% air-dried cooked egg albumen or fibrin and an allowance of B factors ordinarily considered adequate. When 0.6% Ca is added (line 4), growth is good and the stomachs are normal. Without the calcium,

Fig. 2 Single lesions in the rumen along the ridge and at the esophageal opening, the favorite sites for this lesion. One lesion is round, has raised thickened edges and a central depression. The other at the esophagus consists of an irregular wrinkled thickening of the mucosa. The majority of the rumen lesions obtained on diets described in these papers are like those in this picture (cf. fig. 3).

however, 88% of the animals showed antral lesions with a count of 11.9 per rat. There were rumen lesions in 17% of these animals, while the fundus was normal in the sense that only one of the 134 rats showed a mild hemorrhage. The rats fed the second calcium deficient diet (line 3), which contained 27% casein and about twice as much B complex as the

previous one, showed no abnormalities except in the antrum (85% of the rats, with a lesion count of 13.6 per rat).

Among other things the data indicate that the three kinds of lesion have separate causes and that it is possible to produce any one of them in 28 days. Howes and Vivier ('36) treated their data as though lesions

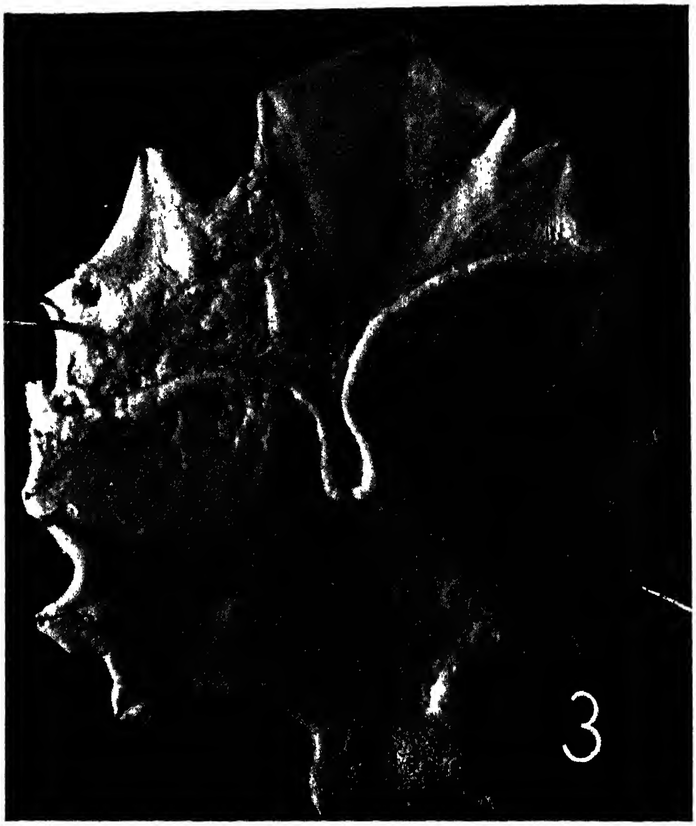


Fig. 3 An unusually extensive effect involving approximately one-half of the lining of the rumen. It consists of nodular thickening with fissures and craters of irregular shapes. The ridge in this specimen shows thickening and has a beaded appearance. This antrum also shows lesions. The furrowed appearance of the fundus is normal and depends on the amount of stretching and state of contraction of the muscles.

of antrum and rumen have the same cause, and Drummond et al. ('38) thought that "32 days is probably too short a period for the appearance of gastric lesions."

FUNDUS

Apparently the only investigators who have described in detail fundic lesions produced in dietary experiments are Schiödt ('35), who attrib-

uted them specifically to B_1 deficiency, and Brunschwig and Rasmussen ('41), who obtained them under various conditions. In our experience 115 out of 366 animals (31%) on various B complex deficient diets for 4 weeks had fundic hemorrhages (this includes the 234 rats on diet 872 in table 1).

TABLE 1

Distribution of the 3 types of lesions on various diets.[1]

	DIET	NO. OF RATS	INCIDENCE			ANTRUM COUNT
			Rumen	Fundus	Antrum	
1	872, B complex deficient, 12% protein	234	128(55%)	30(13%)	156(67%)	4.9
2	Ca deficient, 20% protein	134	23(17%)	1(0.7%)	118(88%)	11.9
3	Ca deficient, 27% protein	141	2(1.4%)	2(1.4%)	120(85%)	13.6
4	Normal, 20% protein	124	1(0.8%)	0	4(4%)	0.5
5	Normal, 27% protein	94	0	0	2(2%)	0.2
6	Stock	198	2(1%)	0	2(1%)	0.2

[1] All experiments were run 4 weeks. For details on scoring the antrum lesions see the preceding paper (Zucker et al., '45).

Single B deficiencies

The question now arose which of the B factors was responsible. The feeding of a control synthetic diet (Stoerk and Zucker, '44) containing liberal allowances of the usual six factors, namely, thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline, with no other known source of B factors and 27% Labco casein as the protein source, resulted in normal stomachs. Five diets, each singly deficient in one of the five factors thiamine, riboflavin, pyridoxine, pantothenic acid and choline, were fed to 4-week-old rats for a period of 4 weeks. The omission of choline led to no discernible effect on growth or any other manifestation. The other deficiencies all affected growth in varying degrees. The results on the stomach can be briefly stated to the effect that fundic lesions were associated with thiamine deficiency only. Such was the case when the respective deficiencies were complete — i.e., zero amounts of the factor in question added. With about one-twentieth of the allotment present in the complete diet no signs of hemorrhage were seen even in the case of thiamine deficiency.

Starvation

This result raised the question whether in the thiamine deficiency experiments the effect was specific for thiamine or whether the inanition

was responsible. Rats allowed the same intake of a normal diet as was spontaneously taken by B deficient animals showed a high incidence of fundic hemorrhage at the end of 3 to 4 weeks. Observations on total food deprivation show that with animals 40 ($\pm$ 1) days old a fast of $2\frac{1}{2}$ days will produce definite if not fatal fundic hemorrhage with a weight loss of 30 to 40%. Attempts were made to prevent this with 1 gm. quantities daily of either cerelose or casein with or without vitamin and mineral supplements of insignificant caloric value. Twenty-five such attempts were made with no noticeable deviation from the control results except that the daily supply of 4 cal. increased survival time by about 1 day.

The trials included: 1 gm. liver powder (Wilson 1:20); 0.5 gm. yeast extract (Standard Brands type 3, equivalent to 1.5 gm. yeast); ascorbic acid (10 mg. in water given twice daily starting 3 days before food deprivation); vitamin K_1 (0.5 mg. per day) in 1 drop of oil; a mixture of 0.15 gm. Wesson salts, ascorbic acid, the six available pure B factors in amounts well above the daily requirement together with 1 gm. of either cerelose or crude casein. As a further check on the possibility that unknown factors play a role a daily allotment of 2.5 gm. fresh feces from stock rats was tried but with no effect. In animals 70 days old and weighing from 230 to 250 gm. about 5 days were required to produce a similar percentage weight loss with less but still quite definite hemorrhage in the fundus.

It appears that the fundic lesions are not related to any one essential food factor as indicated by the various trials with supplements and also by the rapidity with which the striking symptoms appear. They are probably due to a physiological process in response to absence of food, which is apparently more acute in 40 than in 70-day-old animals. It is outside the scope of this paper to look into further mechanisms which may lie in other fields such as vascular or nervous physiology.

Other combinations of several deficiencies, especially if they lead to nearly negligible food intake before the experiment is terminated, may also be responsible for fundic hemorrhage. Profuse hemorrhage in the gastro-intestinal tract has been mentioned incidentally in quite a few nutrition studies (e.g., Sherman and Derbigny, '32), where by inference at least it is related to a particular factor. Fundic hemorrhage can vary from mere petechiae to such brisk bleeding that the small intestine is filled with blood for a good part of its entire length. In these cases the source of hemorrhage is easily determined if the stomach is opened. Such massive hemorrhage may lead to sudden death, but some animals withstand it and show blood, obvious or occult, in the feces. We have

observed fundic hemorrhages in the course of another investigation where with simultaneous kidney involvement the non-protein nitrogen and urea of the blood were high. The association of high non-protein nitrogen in the blood and gastric hemorrhage is not unfamiliar in human pathology.

Effect of protein level

It is not clear why an increase in the protein level in B deficient diets was associated with an increase in the incidence of fundic lesions (12% casein, incidence 13%; 20% casein, incidence 57%; 27% casein, incidence 93%). These lesions are not produced during growth, but within a few days when weight is being lost, and it is possible that the better the animal's nutritive state was with respect to protein prior to the weight loss — i.e., the better it grew initially — the greater the lesion-producing stress when the weight loss occurs. The absence of any notation on fundic lesions in accounts of experiments involving B deficiencies and low protein (those of Sharpless, for example) may thus be accounted for.

Non-nutritional causation of fundic hemorrhage

The identical type of fundic hemorrhage can also be produced by procedures which in no way involve food or food intake level. Dodds et al. ('34) have produced acute hemorrhages which appear a few hours after the injection of pituitary extracts. These investigators emphasize that the effects are strictly limited to the fundic "acid bearing portion of the stomach" of several species including the rat. Hueper's ('27) report on gastric hemorrhages produced by Mathews and Austin in dogs by injection of parathormone shows that here also the fundic region is affected. Dr. Herbert Stoerk of this department has made similar observations on rats, the resulting disturbance being the same as that here reported in case of acute B_1 deficiency or starvation. The parathormone lesions appear long before they are seen in inanition controls.

RUMEN

The changes in the rat's rumen have for some time attracted considerable attention. Pappenheimer and Larimore ('24) reported their presence in rats fed highly deficient diets and suggested that nutritional deficiency played a role in their production. Two mutually exclusive views as to the nature of the deficiency have been advanced, one holding that vitamin A deficiency is the cause while the other relates them to

water soluble factors.[4] There can be no question regarding the finding of hyperplasia of the rumen on A-deficient diets but the conclusion that the lesions are referable to this deficiency in any specific sense can hardly be upheld: the lesions can be produced in the presence of an ample supply of vitamin A, and can be absent even though there is a well established A deficiency. However, in spite of the clear demonstration by Wolbach and Howe ('25; '33) that among the typically reproducible symptoms of vitamin A deficiency these lesions are not found, reports have continued to appear (Fridericia et al., '40; Beck and Peacock, '41) which quite plausibly relate this condition to A deficiency. In cases where the composition of the diet is given in detail it seems impossible to discern significant differences on the basis of known factors: on diets used for A assay, Richards ('35) and Bacharach and Smith ('33) recorded hyperplasia of the rumen while Dyer and Roe ('41) described the rumen as normal. Neither is it the judgment of one investigator as against that of another since Cramer ('37) failed to confirm his own previous findings and was not able to assign a cause for the discrepancy. Any final account of causation, to be satisfactory, must clarify this puzzling situation.

Sharpless has extensively studied hyperplasia of the rumen produced by deficiencies of water soluble factors in the presence of ample vitamin A. According to Sharpless the lesions are not due to a specific deficiency but occur if any one of the following factors is inadequately supplied: cystine, riboflavin, pyridoxine, nicotinic acid, and choline. It appears that Sharpless has gone further than others towards clarification of the problem.

All of our work to date has been pointed specifically at the glandular part of the stomach either antrum or fundus, but the observations on the rumen made in the course of the work are not without interest. We have been able to substantiate the findings of Sharpless on the following points: lesions are found on low casein diets, deficiency of certain B factors will produce them, and vitamin A does not prevent them even when present in more than adequate amounts.

[4] We can pass over two other suggested causes, namely, virus and worms (see Lancet, '38). Another non-nutritional cause was considered established by Büchner et al. ('28), namely, hyperacidity, on the basis of histamine injection. When Henning and Norpoth ('32) supplied the necessary control experiments, however, it was found that stomach secretion of the rat is refractory to histamine (confirmed by Friedman, '43, and by Komrow et al., '44). Brummelkamp ('38) also related the lesions to hyperacidity but did not obtain results when housing the animals in metal cages; wood must be available which the rats gnaw but do not swallow. Our studies do not resolve this point because we use only metal cages.

Prevention with purified casein

We are not convinced, however, that the non-specific causation hypothesis of Sharpless expresses the only interpretation which can be made of the data. Even in the absence of appreciable amounts of the majority of the factors each of which Sharpless holds to be essential, complete prevention can be achieved. This is illustrated in table 2 where a graded increase of purified casein in the diet from 6% to 27% progressively decreases the lesion incidence from 92% to 0%. At first sight it may seem a little incongruous with general experience that 27% of protein should be required to obtain a completely normal rumen, but on this type of diet (B complex deficient) the food intake is so low that the average daily protein ingestion is no more than on a level of

TABLE 2

Evidence that the responsible factor is present in crude and purified casein.[1]

CASEIN LEVEL	CRUDE CASEIN		LABCO CASEIN	
	No. of rats	Incidence of rumen lesions	No. of rats	Incidence of rumen lesions
		%		%
6	29	34	47	92
12	21	0	56	43 [2]
20			43	8 [3]
27			27	0 [4]

[1] These diets contain no added source of B factors; besides the casein they have 2% fat contributing 750 units of vitamin A activity from carotene and 540 from cod liver oil, salts, and carbohydrate (starch, sucrose or cerelose) to 100. Details of diet compositions are given in table 2 of the preceding paper (Zucker et al., '45).

[2] Diet 872 — a group run at the same time as the group on the 12% crude casein diet.

[3] Diets 896 and 896 + bone ash.

[4] Diets 1019 and 1019 + bone ash.

10–12% in an otherwise adequate diet. Howes and Vivier as well as Sharpless found in a few animals that 20% of casein did not prevent the hyperplasia. This is in agreement with the data of our table 2 but we are not ready to agree with their inference that this satisfies all the conditions necessary for establishing the role of casein.

While at one time casein was almost axiomatically considered an ideal protein to use in nutrition studies, this is no longer the case. Its amino acid constitution leaves a good deal to be desired. A relative deficiency in sulfur-containing amino acids has been established, notably by the finding that growth and lactation performance can be definitely improved by supplements of cystine (Daggs et al., '35 a, b; '38; Mulford and Griffith, '42; see also Almquist, '43). Casein is difficult to purify,

as is seen when it is to be used for vitamin A studies (see account by Coward, '38). Observations with highly purified casein led a number of investigators to the conclusion that the removal of the last impurities reveals a new nutritional deficiency related to a factor closely associated with casein and present in all but the most highly purified samples (Bacharach, '33; Bacharach and Smith, '33; Coward, '38; Mapson, '32; '33). This factor can be supplied by liver extract (Mapson, '32; '33; Hartman, Dryden and Cary, '41). Jones and Gersdorff ('34), on the other hand, call attention to the fact that vigorous purification methods (alkali treatment, heat treatment) affect the casein itself in the sense that the low cystine content is still further reduced.

The difference between a sample of crude and purified casein may therefore lie either in its content of cystine (and possibly other amino acids which are labile to the method of purification) or in the amount of unidentified nutritional factors still adhering to it. In both respects the crude casein is likely to have the more favorable nutritional properties. Further study should reveal which of these is responsible for prevention of these lesions on relatively low levels of crude casein.

There will certainly also be a difference between crude and purified casein with respect to content of the known B factors, but this does not appear to be pertinent here in view of the fact that prevention is attained at a level of purified casein on which there is no difficulty in obtaining satisfactory deficiencies of all the known factors (see previous discussion under Fundus).

It may be noted here that none of the single deficiencies of the known factors including choline produced any detectable changes in the rumen in a period of 4 weeks. Neither is there as yet any indication that the 27% casein effect is equivalent to a choline (labile methyl) effect. A group of animals on a B complex deficient diet with 12% Labco casein plus an ample choline supplement showed seven out of nine animals with hyperplastic rumens, as compared with five out of eight in the control animals.

The difference between the results at a given level of feeding with crude as compared with purified casein is not a matter of difference in food intake; the pattern of growth and food intake over the 4 weeks is just about the same in the two cases. For this reason it is to be assumed that the animals on the crude casein diet are getting a larger supply of the preventive factor because there is a higher concentration of it in the crude casein; rough interpolation of the dose-action curve effects of table 2 suggests that "Labco" casein has about one-half as much of the factor as crude casein.

This point of view affords a basis for re-evaluation of the contradictory vitamin A results. Lesions of the rumen on vitamin A deficient diets may be referable to a more drastically purified casein. With a diet supplying a borderline amount of the preventive factor, any other deficiency which lowers the food intake may cause the appearance of lesions. Support for the role of lowered food intake may be found in a number of our observations of which we will cite two examples.

A supplement of 15% of autoclaved yeast added to diet 872 in no way lessened the incidence (13 out of 17 animals) and did not improve growth, while 15% of unautoclaved yeast more than doubled the food intake, changed the growth from a small net loss to a gain of 60 gm. in 4 weeks and prevented the lesions in the rumen (incidence 0 in 10 animals). The second example shows that, in the Sharpless sense, calcium could be added to the list of pertinent factors since, by comparison of lines 2 and 4 of table 1, calcium makes the difference between occurrence and prevention. The 4-week food intakes for these calcium-deficient and calcium-supplemented groups were, respectively, 140 and 230 gm.

SUMMARY

1. While fundic lesions can be produced on deficient diets, their cause probably lies in inanition rather than any specific relation of a food factor to the structure of the fundic mucosa.

2. Since the maintenance of fundic mucosa is thus removed from the narrower field of nutrition, other mechanisms involving other fields of physiology seem indicated (vascular, neurological, etc.).

3. The rat's rumen responds to some deficient diets with a quite reproducible reaction of hyperplasia and hyperkeratosis. While these lesions have been ascribed to deficiencies of both vitamin A and members of the B complex, it is shown that they can be produced in the presence of ample vitamin A in the diet, and prevented on a totally B-deficient diet by increasing the (purified) casein content to 27%.

4. The data suggest the possibility of a specific factor important for the prevention of lesions of the rumen which may be either an amino acid labile to commonly employed methods of purifying casein, or an unknown factor associated with crude casein.

5. To clarify the nature of the postulated specific deficiency the role of both food intake and diet composition will have to be subjected to more exacting study.

LITERATURE CITED

ALMQUIST, H. J. 1943 In "Addendum to the Chemistry of the Amino Acids and Proteins," edited by C. L. A. Schmidt. Charles C. Thomas, Baltimore, p. 1266.

BACHARACH, A. L. 1933 Investigations into vitamin A-free basal diets. I. Biochem. J., vol. 27, p. 5.

BACHARACH, A. L., AND E. L. SMITH 1933 Investigations into vitamin A-free diets. II. Biochem. J., vol. 27, p. 17.

BECK, S., AND P. R. PEACOCK 1941 Gastropapillomatosis due to vitamin A deficiency induced by heated fat. Brit. Med. J., part 2, p. 81.

BONGERT, J. 1912 Ueber die Entstehung des Ulcus pepticum beim Kalbe. Berl. Klin. Woch., S. 807.

BRUMMELKAMP, R. 1938 On the cause of gastric ulcers — an experimental study. Amsterdam.

BRUNSCHWIG, A., AND R. A. RASMUSSEN 1941 The relation of diet to benign neoplasia (ulcero-papillomas) of the rat's stomach. Cancer Res., vol. 1, p. 371.

BÜCHNER, F., P. SIEBERT AND P. J. MOLLOY 1928 Ueber experimentell erzeugte akute peptische Geschwüre des Rattenvormagens. Beiträge z. path. Anat., Bd. 81, S. 391.

COWARD, K. H. 1938 The Biological Standardisation of the Vitamins. William Wood and Co., Baltimore, pp. 24–26.

CRAMER, W. 1937 Papillomatosis in the forestomach of the rat and its bearing on the work of Fibiger. Am. J. Cancer, vol. 31, p. 537.

DAGGS, R. G. 1935 a Studies on lactation. II. Technic for studying lactation in rats. J. Nutrition, vol. 9, p. 575.

DAGGS, R. G., AND R. L. TOMBOULIAN 1935 b Studies on lactation. III. Effect of various dietary principles on lactation in rats. J. Nutrition, vol. 9, p. 581.

DAGGS, R. G., AND V. S. M. LIDFELDT 1938 The effect of the sulfhydryl compounds on milk production. J. Nutrition, vol. 15, p. 211.

DODDS, E. C., R. L. NOBLE AND E. R. SMITH 1934 A gastric lesion produced by an extract of the pituitary gland. Lancet, part 2, p. 918.

DRUMMOND, J. C., A. Z. BAKER, M. D. WRIGHT, P. M. MARRIAN AND E. M. SINGER 1938 The effect of life-long subsistence on diets providing sub-optimal amounts of the "vitamin B complex." J. Hygiene, vol. 38, p. 356.

DYER, H. M., AND J. H. ROE 1941 The relation of nutrition to gastric function. IV. The effect of vitamin A deficiency. Am. J. Dig. Dis., vol. 8, p. 329.

FRIDERICIA, L. S., S. GUDJONSSON, B. VIMTRUP, S. CLEMMESEN AND J. CLEMMESEN 1940 Stomach lesions in rats kept on diets deficient in vitamin A. Am. J. Cancer, vol. 39, p. 61.

FRIEDMAN, M. F. H. 1943 Histamine ineffective in the rat as a gastric secretory stimulant. Proc. Soc. Exp. Biol. Med., vol. 54, p. 42.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1941 Deficiencies other than vitamin A in vitamin A-free diets. J. Biol. Chem., vol. 140, p. liv. Further details in 1941 and 1942 Reports of the Chief of the Bureau of Dairy Industry, U. S. Dept. Agriculture.

HENNING, N., AND L. NORPOTH 1932 Untersuchungen über den Selbstschutz des Magens gegen Salzsäureverätzung. Arch. f. exp. Path. u. Pharm., Bd. 167, S. 224.

HOWES, E. L., AND P. J. VIVIER 1936 The relation of diet to the occurrence of gastric lesions in the rat. Am. J. Path., vol. 12, p. 689.

HUEPER, W. 1927 Metastatic calcification in the organs of the dog after injection of parathyroid extract. Arch. Path., vol. 3, p. 14.

JONES, D. B., AND C. E. F. GERSDORFF 1934 The effect of dilute alkali on the cystine content of casein. J. Biol. Chem., vol. 104, p. 99.

KOMROW, S. A., H. SHAY, D. RAPPORT AND S. S. FELS 1944 Some observations on gastric secretion in normal rats. Gastroenterology, vol. 3, p. 406.

KONJETZNY, G. E., AND H. PUHL 1926 Das sogenannte Ulcus pepticum der Absatzkälber. Virchow's Arch., Bd. 262, S. 615.

LANCET 1938 Fibiger's tumor of the rat's stomach (Annotations). Page 735.

MAPSON, L. W. 1932 Evidence of the existence of a dietary principle stimulating growth and lactation. Biochem. J., vol. 26, p. 970.

——— 1933 A deficiency disorder induced in suckling young rats bred on a purified synthetic diet with "Glaxo casein" (caseinogen) as sole source of protein. Biochem. J., vol. 27, p. 1061.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. VIII. The relation of cystine and methionine to the requirement of choline in young rats. J. Nutrition, vol. 23, p. 91.

PAPPENHEIMER, A. M., AND L. D. LARIMORE 1924 The occurrence of gastric lesions in rats. Their relation to dietary deficiency and hair ingestion. J. Exp. Med., vol. 40, p. 719.

RICHARDS, M. B. 1935 The role of vitamin A in nutrition. Brit. Med. J., vol. 1, p. 99.

SCHIÖDT, E. 1935 Gastro-intestinal affections from vitamin deficiency. Acta Med. Scand., vol. 84, p. 456.

SHARPLESS, G. R. 1936 Neoplastic hyperplasia in gastric mucosa of rats. Proc. Soc. Exp. Biol. Med., vol. 34, p. 684.

——— 1937 Malignant changes in the forestomach of rats related to low protein (casein) diet and prevented by cystine. Ann. Surgery, vol. 106, p. 562.

——— 1940 a Prevention of hyperplasia in the forestomach epithelium of rats fed white flour. J. Nutrition, vol. 19, p. 31.

——— 1940 b Choline and epithelial hyperplasia in the forestomach of rats. Proc. Soc. Exp. Biol. Med., vol. 45, p. 487.

——— 1943 a Diet and epithelial hyperplasia in the forestomach of rats and mice. Cancer Res., vol. 3, p. 108.

SHARPLESS, G. R., AND M. SABOL 1943 b Choline and pyridoxine as factors in prevention of epithelial hyperplasia in the forestomach of rats fed white flour. J. Nutrition, vol. 25, p. 113.

SHERMAN, H. C., AND I. A. DERBIGNY 1932 Studies on vitamin G (B_2) with special reference to protein intake. J. Biol. Chem., vol. 99, p. 165.

STOERK, H. C., AND T. F. ZUCKER 1944 Nutritional effects on the development and atrophy of the thymus. Proc. Soc. Exp. Biol. Med., vol. 56, p. 151.

TANTZ, A. 1912 Beitrag zum Vorkommen, zur Ätiologie und zur fleischbeschaulichen Beurteilung des Ulcuspepticum beim Rinde. Inaug. Diss. Giessen.

WOLBACH, S. B., AND P. R. HOWE 1925 Tissue changes following deprivation of fat-soluble A vitamin. J. Exp. Med., vol. 42, p. 753.

——— 1933 Epithelial repair in recovery from vitamin A deficiency. J. Exp. Med., vol. 57, p. 511.

ZUCKER, T. F., B. N. BERG AND L. M. ZUCKER 1945 Nutritional effects on the gastric mucosa of the rat. I. Lesions of the antrum. J. Nutrition, vol. 30, p. 301.

THE EFFECTS OF THE DIETARY SUPPLY OF CARBOHYDRATE UPON THE RESPONSE OF THE HUMAN RESPIRATORY QUOTIENT AFTER GLUCOSE ADMINISTRATION

HOWARD F. ROOT AND THORNE M. CARPENTER

The George F. Baker Clinic,[1] *New England Deaconess Hospital and the Nutrition Laboratory, Carnegie Institution of Washington, Boston*

(Received for publication July 5, 1944)

The effects of the character of the preceding diet, especially with reference to fat and carbohydrate contents, upon the respiratory quotient of man has been studied by a number of workers (Frentzel and Reach, '01; Benedict, Emmes and Rich, '11; Benedict and Higgins, '12; Krogh and Lindhard, '20; Marsh, '28; Marsh and Murlin, '28; Bierring, '31; Hitchcock, '34; Sheldon, Johnson, and Newburgh, '37; Talbott, Coombs, Consolazio, and Pecora, '38). In only one of these studies (Sheldon and coworkers) was the effect of the preceding diet upon the combustion of glucose given as a dose at the beginning of the experiment determined. These workers gave diets containing 21 to 500 gm. of carbohydrates and measured the respiratory quotient (R.Q.) and the combustion of carbohydrates for 4 hours on days following these diets. In another series they measured these factors after giving 25 to 200 gm. of glucose preceded by different dietary levels of carbohydrates, but they did not make a basal determination on the day on which the glucose was given. They found that the carbohydrate level of the preceding day's diet influenced the height of the R.Q. on basal days and on the days on which glucose was given and that the effects on the carbohydrate combustion were additive, that is, the rise in basal R.Q. due to high carbohydrate intake and the rise due to carbohydrate given at the beginning of the day's measurements were summated when carbohydrates were given after high carbohydrate diets on the preceding days.

The purpose of this study was to determine the effect of the level of carbohydrates in the daily diet on the increase in the respiratory quotient (R.Q.) and on the combustion of carbohydrates after the ingestion orally of 50 gm. of glucose when the subject was in the basal condition.

[1] Dr. Elliott P. Joslin, Medical Director.

Two medical students, F. B., 26 years old, 73.0 kilos without clothes, and 179 centimeters in height, and C. J. R., 25 years old, 59.5 kilos without clothes and 173 centimeters in height, were placed on a prescribed diet for several days at definite levels of intake of carbohydrates and the basal respiratory exchange and the respiratory exchange after oral ingestion of 50 gm. of glucose was determined by the open circuit method with a Benedict (Benedict, '33) helmet and gas analysis (Carpenter, '33) in twelve successive 15-minute periods on every other day.

TABLE 1

Daily diet of F. B. and C. J. R.

DATE 1940	F. B.					C. J. R.				
	P	F	C	Cal.	R.Q	P	F	C	Cal.	R.Q.
	gm.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*		
June 8	87	47	349	2167	0.91	. .	. .	. . .	. .	. . .
June 9	87	47	349	2167	0.91	87	47	349	2167	0.91
June 10	75	63	344 [1]	2243	0.89	87	47	349	2167	0.91
June 11	91	70	349	2475	0.89	69	57	335 [1]	2129	0.90
June 12	85	75	335 [1]	2355	0.88	87	47	349	2167	0.91
June 13	87	63	329	2231	0.89	73	81	335 [1]	2361	0.88
June 14	[2]	[2]	[2, 1]	[2]	[2]	87	73	349	2401	0.89
June 15	85	185	87	2353	0.76	[2]	[2]	[2, 1]	[2]	[2]
June 16	83	185	75	2297	0.76	83	197	75	2405	0.76
June 17	108	174	77 [1]	2306	0.77	83	197	75	2405	0.76
June 18	89	191	84	2411	0.76	107	186	76 [1]	2406	0.76
June 19	108	174	77 [1]	2306	0.77	83	197	75	2405	0.76
June 20	83	185	75	2297	0.76	107	186	76 [1]	2406	0.76
June 21	108	178	50 [1]	2234	0.75	83	197	75	2405	0.76
June 22	107	178	11	2074	0.74	108	187	50 [1]	2315	0.75
June 23	107	178	11	2074	0.74	110	184	11	2140	0.74
June 24	108	178	50 [1]	2234	0.75	110	184	11	2140	0.74
June 25	. . .	. . .	. . .	. . . .	. . .	108	187	50 [1]	2315	0.75
June 26	. .	. .	. . .	. . . .	. .	107	184	0	2084	0.73

[1] Fifty gm. glucose instead of breakfast.

[2] Diet for the day chosen by the subject. No record made.

The amounts of protein, fat, carbohydrates, calories, and the calculated R.Q.'s in the daily diet are given in table 1. The theoretical R.Q. of the dietary intake was calculated from the usual factors for respiratory exchange on the assumption that the fat and a mixture of carbohydrates (starch and sugar) was completely oxidized and that the protein gave 774 ml. of carbon dioxide and required 957 ml. of oxygen per gram for the portion of the protein burned (Carpenter, '39). There were three levels of carbohydrates in the diets namely, approximately 350 gm., 75 gm., and 11 gm., respectively. Table 1 included the 50 gm. of glucose on

the days on which this amount was given. The R.Q.'s of the diets calculated from the components show that at the high level, the R.Q.'s varied from 0.88 to 0.91 and at the 75-gm. level, from 0.76 to 0.77. The lowest R.Q. of the diet, 0.73, was on June 26, with C. J. R., with no carbohydrates in the diet.

The body weights of the subjects fell from 73.2, with F. B., to 70.0 kilos and with C. J. R., from 59.1 to 57.4 during the series. They continued their physical activity in hospital wards and medical school throughout the period.

TABLE 2

Analyses of blood and urine.[1]

DATE AND SUBJECT	CARBOHYDRATE LEVEL	TIME	URINE SUGAR	ACETONE	DIACETIC ACID	BLOOD SUGAR
	gm.		%			%
F. B.						
June 10	350	Fasting	0	—	0	0.09
		After sugar	0.2	—	—	0.07
June 21	75	Fasting	tr	0	0	0.09
		After sugar	0.3	tr	0	0.07
June 24	11	Fasting	0.3	tr	0	0.07
		After sugar	0.4	+	0	0.06
C. J. R.						
June 15	350	Fasting	0	—	0	0.09
		After sugar	0	—	0	0.07
June 22	75	Fasting	0.2	—	0	0.08
		After sugar	0	—	0	0.07
June 25	11	Fasting	0	tr	0	0.09
		After sugar	0.4	+	0	0.09
June 27	0	Fasting	0	+	0	0.08
		After sugar	0	tr	0	0.09

[1] Where dashes occur in the table, no determinations were made.

The urines were collected immediately on arrival at the Laboratory in the morning and then immediately after the series of determinations on the R.Q.'s were made after sugar. Blood samples were also taken about one-half hour after the first urine was voided and at the end of the experimental periods at about 12:30 P.M. The urines were tested for sugar, acetone, and diacetic acid in most cases, and the blood sugar was determined, but unfortunately the urinary nitrogen was not determined. Sample results at the different levels of carbohydrate intake in which the amounts given represent the diet of the day before the test are given in table 2. The striking fact is that with the marked changes in carbohydrate there were practically no changes in the blood sugar. The lowest level is with F. B. on June 24 when it fell to 0.06% in the sample after

the sugar was taken. Usually, the blood sugar was lower at noon than in the fasting condition by 0.01 to 0.02%. When the carbohydrate intake was reduced to only 11 gm. the administration of glucose was not followed by as marked a fall in the blood sugar with C. J. R. as previously. It may be supposed that with a low carbohydrate intake less insulin was made and there was a slighter response by the outpouring of insulin after giving the 50 gm. of glucose than was true on the higher diet. Urine analyses were for the most part negative and acetone appeared only in traces in the case of F. B. on June 21 and June 24 and similarly on June 25 and June 27 with C. J. R. Nothing resembling clinical acidosis occurred even when the subjects were on only 11 gm. carbohydrate and 197 gm. fat.

The changes in the R.Q. from the base line for 3 hours after the ingestion of 50 gm. of glucose are given in table 3. With F. B., the base line's R.Q.'s after 350 gm. of carbohydrates were much lower than the calculated R.Q. of the diet so that from the time the last meal was ingested until the base line measurement was made, there was a marked fall. This fall may be due to the increased combustion of body fat as the diet was inadequate as shown by the fact that both subjects lost weight during these series of observations. The calculated R.Q. of the diet does not imply that that is necessarily the expected basal R.Q., but was made to show a comparison between the calculated R.Q.'s of the diet and those actually found in the basal condition.

The ingestion of 50 gm. of glucose was followed by a marked rise in R.Q. for the entire 3 hours with an average maximum rise of 0.10 in the seventh and eighth 15-minute periods. The net average increases in R.Q. for the 3 hours were 0.08 and 0.06 for the 3 days on the 350-gm. level of carbohydrate intake. When F. B. was on 75 to 87 gm. level in the daily diet, the base line R.Q.'s were nearly the same as the calculated R.Q.'s of daily diet. All three experiments with 50 gm. of glucose showed rises in R.Q. but not so large as with the 350 gm. level of dietary carbohydrates. The net average rise for 3 hours for each of the 3 days was 0.04, thus definitely lower than when the subject was on 350 gm. level of dietary carbohydrate intake. On June 24, after 2 days with 11 gm. of carbohydrates, the base line R.Q. was 0.72, but in spite of this, the rise in R. Q. after ingestion of 50 gm. of glucose was as high as when the subject was on the 75 gm. daily carbohydrate level. The net average 3-hour increase was 0.05.

With C. J. R., the general findings were not so definite. The base line R.Q.'s were not so low and on the 75 gm. carbohydrate daily dietary level, the base line R.Q.'s were higher on 2 days than those calculated

TABLE 3

Changes in the respiratory quotient after oral ingestion of 50 gm. of glucose as affected by the preceding diet.

DATE AND CONDITION 1940	BASAL R.Q.	CHANGES FROM BASAL IN R.Q. (15-MINUTE PERIODS). 1	2	3	4	5	6	7	8	9	10	11	12
F. B.													
350 gm. carbohydrate													
June 10	0.77	− 0.01	+ 0.03	+ 0.07	+ 0.09	+ 0.11	+ 0.10	+ 0.12	+ 0.13	+ 0.11	+ 0.10	+ 0.02	+ 0.08
June 12	0.80	− 0.01	+ 0.01	+ 0.08	+ 0.09	+ 0.08	+ 0.07	+ 0.09	+ 0.09	+ 0.08	+ 0.08	+ 0.03	+ 0.06
June 14	0.79	− 0.03	+ 0.02	+ 0.08	+ 0.10	+ 0.08	+ 0.09	+ 0.09	+ 0.07	+ 0.07	+ 0.06	+ 0.05	+ 0.01
75 gm. carbohydrate													
June 17	0.76	− 0.02	− 0.01	+ 0.03	+ 0.04	+ 0.04	+ 0.07	+ 0.08	+ 0.08	+ 0.08	+ 0.03	+ 0.04	+ 0.05
June 19	0.77	− 0.02	− 0.01	+ 0.05	+ 0.06	+ 0.05	+ 0.05	+ 0.08	+ 0.08	+ 0.04	+ 0.03	+ 0.02	+ 0.03
June 21	0.75	− 0.01	+ 0.01	+ 0.02	+ 0.04	+ 0.03	+ 0.05	+ 0.09	+ 0.07	+ 0.06	+ 0.05	+ 0.07	+ 0.02
0–11 gm. carbohydrate													
June 24	0.72	− 0.01	+ 0.01	+ 0.03	+ 0.02	+ 0.06	+ 0.08	+ 0.08	+ 0.08	+ 0.09	+ 0.06	+ 0.08	+ 0.04
C. J. R.													
350 gm. carbohydrate													
June 11	0.84	− 0.05	− 0.01	+ 0.02	+ 0.05	+ 0.05	+ 0.07	+ 0.10	+ 0.08	+ 0.05	+ 0.07	+ 0.07	+ 0.09
June 13	0.80	− 0.01	+ 0.02	+ 0.07	+ 0.05	+ 0.05	+ 0.05	+ 0.10	+ 0.05	+ 0.09	+ 0.03	+ 0.03	+ 0.07
June 15	0.82	− 0.04	+ 0.01	+ 0.04	+ 0.06	+ 0.06	+ 0.06	+ 0.04	+ 0.09	+ 0.07	+ 0.07	+ 0.05	+ 0.07
75 gm. carbohydrate													
June 18	0.75	− 0.02	+ 0.02	+ 0.04	+ 0.05	+ 0.05	+ 0.05	+ 0.07	+ 0.08	+ 0.08	+ 0.07	+ 0.07	+ 0.08
June 20	0.78	− 0.02	− 0.03	0.00	+ 0.01	+ 0.01	+ 0.04	+ 0.03	+ 0.06	+ 0.06	+ 0.06	+ 0.01	+ 0.01
June 22	0.78	− 0.03	− 0.01	0.00	+ 0.01	+ 0.03	+ 0.04	+ 0.06	+ 0.06	+ 0.05	+ 0.03	+ 0.06	+ 0.03
0–11 gm. carbohydrate													
June 25	0.75	0.00	− 0.01	+ 0.02	0.00	+ 0.02	+ 0.05	+ 0.04	+ 0.07	+ 0.06	+ 0.04	+ 0.05	+ 0.08
June 27	0.74	− 0.03	− 0.01	0.00	+ 0.03	0.00	+ 0.02	+ 0.03	+ 0.05	0.00	+ 0.03	+ 0.01	+ 0.06

from dietary intake. The net average rise in the R.Q. for 3 hours on each of the 3 days on the 350 gm. carbohydrate intake was 0.05 and on the 3 days with 75 gm. for dietary carbohydrate, the net average rises for 3 hours in R. Q. were 0.05, 0.02, and 0.03, and on the lowest carbohydrate intake, were 0.04 and 0.02.

The increases in the heat production and in the combustion of carbohydrates are given in table 4. The heat production in the base line

TABLE 4

Increase in heat production and combustion of carbohydrates after ingestion of 50 gm. of glucose (values for 3 hours).

DATE 1940	BASE LINE		INCREASE AFTER 50 GM. GLUCOSE	
	Heat production	Carbohydrate burned	Heat production	Carbohydrate burned
	cal.	*gm.*	*cal.*	*gm.*
F. B.				
350 gm. carbohydrate				
June 10	229.6	8.1	13.8	16.3
June 12	237.8	15.2	6.3	12.8
June 14	229.7	12.7	19.5	13.0
75 gm. carbohydrate				
June 17	215.4	5.9	25.2	9.1
June 19	226.7	7.1	9.1	8.9
June 21	236.4	5.4	3.2	8.1
11 gm. carbohydrate				
June 24	220.0	0.0	13.5	7.9
C. J. R.				
350 gm. carbohydrate				
June 11	182.8	16.1	12.8	9.0
June 13	184.1	10.2	15.9	9.2
June 15	186.4	14.0	5.9	7.8
75 gm. carbohydrate				
June 18	202.3	4.4	3.3	8.6
June 20	190.8	8.8	12.9	4.0
June 22	191.7	8.1	9.2	5.1
0–11 gm. carbohydrate				
June 25	194.8	2.5	8.8	6.2
June 27	188.8	1.6	16.6	3.4

periods of F. B. on the 350 gm. intake of carbohydrates averaged 232.4 cal., on the 75 gm., 226.2, and on the lowest, on 1 day, 220.0 cal. There is no evidence with this subject that the varying levels of carbohydrate intake affected the base line heat production. The increase in heat production due to the ingestion of 50 gm. of glucose averaged 13.2, 12.5, and 13.5 cal. for the three levels, but there is considerable variation so that this agreement is only fortuitous. With C. J. R., the average base

line heat production with 350 gm. carbohydrate intake was 184.4, and with the 75 gm., 194.9 calories, and this difference is significant. Part of this increase may have been due to a higher protein level of the diet on the days preceding the measurements, although no data on the nitrogen elimination in the urine are available. On the lowest level it was 191.8. This average is not considered as a significant increase from that on the 350 gm. level. The average increases in the heat production due to 50 gm. of glucose were 11.5, 8.5, and 12.7 cal. in the three series with considerable variation in the individual values.

The combusion of carbohydrates was calculated in the usual empirical manner from the respiratory exchange and an assumed combustion of protein. The protein combustion was calculated from the amount of protein in the preceding day's diet and a coefficient of digestibility of 90%. An actual determination of the nitrogen elimination in the urine would have been better, but it is believed that for comparative purposes the above assumption is justifiable. That changes in carbohydrate combustion after glucose ingestion represent increased carbohydrate combustion is shown by a recent study (Edwards, Bensley, Dill and Carpenter, '44) in which the lactic acid and the alveolar carbon dioxide were determined before and after the ingestion of glucose in man.

The base line carbohydrate combustion with F. B. averaged in the three series 12.0, 6.1, and 0 gm. for the 3 hours. There was thus a definite effect of the carbohydrate intake of the preceding day upon the carbohydrate combustion in the base line condition. After ingestion of 50 gm. of glucose there was an average increase during the 3 hours of 14.0, 8.7, and 7.9 in the three different types of experiments. Thus, the shift from the level of 350 gm. of carbohydrate to one of 75 gm. on the preceding day lowered the increase in carbohydrate combustion for 3 hours following ingestion of 50 gm. of glucose by 5.3 gm. When little or no carbohydrate was taken on the preceding day, there was still an increase of 7.9 gm. in the 3 hours following ingestion of 50 gm. of glucose. The base line carbohydrate combustion of C. J. R. for the 3 hours was 13.4 when on the 350 gm. of carbohydrate level, 7.1 gm. on the 75 gm. level, and 2.1 on the 0 to 11 gm. level. The difference between the first two series, 6.3 with C. J. R., was nearly the same as with F. B., 5.9 gm. This subject showed in all three series the effect of the preceding day's diet on the carbohydrate combustion in the basal condition on the following morning. The increase in the combustion of carbohydrates for 3 hours with this subject after the ingestion of 50 gm. of glucose was 8.7 gm. on the 350 gm. dietary level, 5.9 on the 75 gm. level, and 4.8 on the 0 to 11 gm. level. The differences in the increases in carbohydrate combustion

after 50 gm. of glucose were not so large when C. J. R. was at the three different levels of dietary carbohydrate intake as with F. B. C. J. R. was lighter in weight and had a lower metabolism than F. B. and therefore, C. J. R.'s need of carbohydrate was less than that of F. B. When C. J. R. was on the 350 gm. dietary level, two of the three base line R.Q.'s were higher than those of F. B., but in spite of this, the increase in carbohydrate combustion was not so great with C. J. R. as with F. B. Judging from these two subjects, one may suggest that the amount of increase in carbohydrate combustion after 50 gm. of glucose varies with individuals and that given a certain basal level in one subject, one may not expect the same rise after 50 gm. of glucose in another individual with the same basal level of carbohydrate combustion. It does not follow that the subject who has the highest basal carbohydrate combustion will have the greatest increase in carbohydrate combustion after ingestion of glucose. The level of basal carbohydrate combustion does affect the increase in carbohydrate combustion after the ingestion of glucose but even when the basal combustion reaches zero, the normal individual is still able to burn some carbohydrate in spite of the need to augment the glycogen supply. One might expect that with so drastic a lowering of carbohydrate intake with undiminished physical activity the need for deposition of glycogen would be so great that all the glucose would be laid down as glycogen and none would be burned. It is apparent that mere reduction of glycogen does not prevent oxidation of carbohydrate when, as in the normal individual, abundant insulin is present.

SUMMARY

Two medical students were placed on diets for several days at three levels of intake of carbohydrates, namely approximately 350, 75 and 0—11 gm. per day, respectively. The respiratory exchange was measured on alternate days in the basal condition and for 3 hours after the ingestion of 50 gm. of glucose. The combustion of carbohydrates was calculated in the usual empirical manner from the respiratory exchange and the protein level of the day's diet preceding the determinations of the respiratory exchange.

The basal combustion of carbohydrates calculated to 3 hours averaged 12.0, 6.1, and 0 gm. for one subject, and 13.4, 7.1, and 2.1 gm. for the other subject for the three dietary levels.

The increase in combustion of carbohydrates after the ingestion of 50 gm. of glucose with one subject was 14.0, 8.7 and 7.9 gm. and for the other subject, 8.7, 5.9 and 4.8 at the three dietary carbohydrate levels.

Thus the level of carbohydrates in the preceding diet affected the basal combustion of carbohydrates noticeably, and to some extent the increases in carbohydrate combustion after ingestion of glucose, but not so much as one would expect when the demand for replacement of glycogen supply is considered.

The respiratory exchange measurements were made by Mr. Basil James and the diets were under the supervision of Miss Rosina Vance.

LITERATURE CITED

BENEDICT, F. G. 1933 Der Helm-Respirationsapparat in seinen verschiedenen Formen. Abderhalden's Handbuch d. biolog. Arbeitsmethoden, Abt. IV, Teil 13, S. 465.

BENEDICT, F. G., L. E. EMMES AND J. A. RICHE 1911 The influence of the preceding diet on the respiratory quotient after active digestion has ceased. Am. J. Physiol., vol. 27, p. 383.

BENEDICT, F. G., AND H. L. HIGGINS 1912 The influence on the respiratory exchange of varying amounts of carbohydrate in the diet. Am. J. Physiol., vol. 30, p. 217.

BIERRING, E. 1932 The respiratory quotient and the efficiency of moderate exercise (measured in the initial stage and in the steady state during postabsorptive conditions). With special reference to the influence of diet. Arbeitsphysiol., vol. 5, p. 17.

CARPENTER, T. M. 1933 Ein Apparat zur Analyse von Gasen aus Respirationskammern für Menschen und Tiere. Abderhalden's Handbuch d. biolog. Abt. IV, Teil 13, S. 593.

——— 1939 Tables, factors and formulas for computing respiratory exchange and biological transformations of energy. Carnegie Institution of Washington, Publication no. 303B, p. 124.

EDWARDS, H. T., E. H. BENSLEY, D. B. DILL AND T. M. CARPENTER 1944 Human respiratory quotients in relation to alveolar carbon dioxide and blood lactic acid after ingestion of glucose, fructose, or galactose. J. Nutrition, vol. 27, p. 241.

FRENTZEL, J., AND F. REACH 1901 Untersuchungen zur Frage nach der Quelle der Muskelkraft. Pflüger's Arch. f. ges. Physiol., Bd. 83, S. 477.

HITCHCOCK, F. A. 1934 The effect of the preceding diet on the basal respiratory quotient and oxygen consumption. J. Nutrition, vol. 7, Proc. p. 11.

KROGH, A., AND J. LINDHARD 1920 The relative value of fat and carbohydrate as sources of muscular work. Biochem. J., vol. 14, p. 290.

MARSH, M. ELIZABETH 1928 The character of energy metabolism during work. J. Nutrition, vol. 1, p. 57.

MARSH, M. E., AND J. R. MURLIN 1928 Muscular efficiency on high carbohydrate and high fat diets. J. Nutrition, vol. 1, p. 105.

SHELDON, J. M., M. W. JOHNSON AND L. H. NEWBURGH 1937 A quantitative study of the oxidation of glucose in normal and diabetic men. J. Clin. Invest., vol. 16, p. 933.

TALBOTT, J. H., F. S. COOMBS, W. V. CONSOLAZIO AND L. J. PECORA 1938 Respiratory exchange during high carbohydrate ingestion. Am. J. Physiol., vol. 124, p. 246.

BODILY STORAGE OF VITAMIN A IN RELATION TO DIET AND AGE, STUDIED BY THE ASSAY METHOD OF SINGLE FEEDINGS [1]

H. L. CAMPBELL, MADELINE UDILJAK, HELEN YARMOLINSKY AND H. C. SHERMAN

Department of Chemistry, Columbia University, New York

(Received for publication June 27, 1945)

Independent investigations in several laboratories have shown that vitamin A has important relations to nutritional wellbeing at all ages and that the body has powers, when its nutritional intake is ample, to lay up in the liver considerable stores of this vitamin for future use. The experiments recorded in this and the accompanying paper (Caldwell, MacLeod, and Sherman, '45) were designed to develop the quantitative knowledge of this storage function.

PLAN AND METHODS

The plan was to feed well matched young rats, of like hereditary and nutritional backgrounds, from the age of 28 days upon diets, alike in other respects, but of three levels of vitamin A value, namely, 3, 6, and 12 International Units, respectively, of vitamin A per gram of air-dry food. These levels may also be stated as 0.8, 1.6, and 3.2 I.U. of vitamin A, respectively, per Calorie of food. (The laboratory record numbers of these three diets are Nos. 16, 360, and 361, respectively.) The extra vitamin A was introduced in the form of highly potent codliver oil. The offspring of these original experimental animals were continued on their respective family diets until killed for determination of the vitamin A contents of their livers at the ages of 30, 60, 90, 150, 225, and 300 days.

In this study liver storage is believed to be sufficiently indicative of bodily storage, because previous investigations in other laboratories as well as our own have shown the quantities stored elsewhere to be negligible compared with those found in the liver (Sherman and Boynton, '25; Baumann, Riising, and Steenbock, '34; Kao and Sherman, '40; Little, Thomas, and Sherman, '43; Rohrer and Sherman, '43.)

[1] Aided by grants from the Carnegie Institution of Washington and from The Nutrition Foundation, Inc.

TABLE 1

Vitamin A per gram of liver.

AGE	DIET 16 (WITH 3 I.U./GM.)		DIET 360 (WITH 6 I.U./GM.)		DIET 361 (WITH 12 I.U./GM.)	
	Males	Females	Males	Females	Males	Females
28 days						
Mean ± its P.E.[1]	5.7 ± 1.23	4.7 ± 1.17	44.8 ± 3.15	34.3 ± 2.77	157.3 ± 19.32	144.7 ± 13.27
C.V.[2]	117	146	35	42	37	43
Number of cases	13	15	11	12	4	10
60 days						
Mean ± its P.E.	6.8 ± 1.53	5.7 ± 2.02	71.3 ± 6.74	71.7 ± 5.16	177.3 ± 9.21	204.3 ± 8.07
C.V.	126	184	47	40	27	21
Number of cases	14	12	11	14	12	12
90 days						
Mean ± its P.E.	2.7 ± 0.57	8.7 ± 1.05	64.8 ± 2.33	77.0 ± 5.19	244.0 ± 6.12	300.5 ± 6.71
C.V.	114	60	18	38	13	12
Number of cases	13	11	11	14	12	12
150 days						
Mean ± its P.E.	10.8 ± 1.40	19.8 ± 2.24	115.2 ± 4.61	139.1 ± 6.43	379.4 ± 12.67	472.1 ± 15.06
C.V.	67	57	23	26	18	17
Number of cases	12	11	14	14	13	13
225 days						
Mean ± its P.E.	2.1 ± 0.77	4.0 ± 1.14	133.8 ± 5.07	183.8 ± 7.78	604.6 ± 18.29	751.7 ± 26.07
C.V.	198	142	20	22	15	18
Number of cases	13	11	12	12	11	12
300 days						
Mean ± its P.E.	2.1 ± 0.49	13.9 ± 2.33	189.5 ± 14.53	253.5 ± 15.69	783.5 ± 36.67	838.1 ± 42.14
C.V.	122	91	38	35	24	25
Number of cases	12	13	11	14	12	12

[1] Probable error.

[2] Coefficient of variation.

The sexes were caged separately from the age of 28 days. Livers were removed from the experimentally-fed animals and sampled for assay in the manner described in the accompanying paper (Caldwell, MacLeod, and Sherman, '45), and their vitamin A values determined by the single-feeding method developed by Todhunter (Sherman and Todhunter, '34), with separate male and female controls.

EXPERIMENTAL DATA

Table 1 shows the vitamin A values in International Units per gram of liver, averaged separately by age and by sex, each mean value accompanied by its probable error (P.E.), coefficient of variation (C.V.), and number of cases.

TABLE 2

Vitamin A found in livers of rats (from Diets 360 and 361) expressed as approximate percentages of the total potential surpluses which they had received in their food.

AGE	ON DIET WITH 6 UNITS PER GM.		ON DIET WITH 12 UNITS PER GM.	
	Males	Females	Males	Females
60 days	23.0	22.8	28.8	29.5
90 days	10.7	10.5	20.8	21.1
150 days	8.8	10.2	15.8	17.6
225 days	6.2	7.8	15.0	16.3
300 days	7.0	8.9	12.6	13.2

The extremely small amounts found in the livers of the rats whose food contained 3 units per gram are probably best considered as approximately the minimal amounts or concentrations essentially involved in normal maintenance processes. We may consider that this level of nutritional intake supplies approximately the actual (or minimal normal) needs but provides practically no reserve surplus. This may be used as a base-line for an approximate calculation of the quantitative relations of the surpluses offered by the diets of richer vitamin A content and the amounts of stored (reserve) surplus found at different ages in the livers of the animals which had received these diets. The results of these calculations are shown in table 2.

DISCUSSION

Although some observations and discussions tend to throw emphasis upon the fact that surpluses received by the body are not always taken up quickly by the liver (Review, '43), the experiments here reported show that the liver continues over at least a good proportion of the normal life cycle to store a very significant part of surpluses furnished

by intakes two and four times as liberal as the commonly recommended allowances for normal nutrition.

As explained in the preceding section, a level of intake of 3 I.U. of vitamin A per gram of air-dry food, or 0.8 I.U. per Calorie, sufficed for normal growth and maintenance but not to provide for any significant reserve store. The amounts found in the livers of healthy rats on this level at all ages from end of infancy to middle age showed very high coefficients of variation (57 to 198) but this is chiefly because the actual concentrations were so small as to be practically negligible (usually less than 10 I.U. per gram).

At a level of intake twice as high (1.6 I.U. per Calorie) the coefficients of variation were of the order of 20 to 40. With this greater regularity of results, it is doubtless significant that at this level of intake the body increases its reserve store of vitamin A from the end of infancy to middle age, — from 28–30 days to 300 days in the rat. This trend, however, is not quantitatively uniform. The stores found at the age of 90 days show in the females a slackening in the otherwise progressive rate, and in the males a temporary recession; both sexes, however, showing resumption of the upward trend before the age of 150 days. In the experiments of Little who used an entirely different assay method, the data while fewer in number also show a slackening of the storage trend around the age-range of 90 to 150 days (Little, Thomas, and Sherman, '43). As a similar slackening of storage at about 90 days on this same level of intake appears also in the data found simultaneously by a third method of assay (Caldwell, MacLeod, and Sherman, '45), the finding, although not striking, is probably significant. Its significance appears to be that in the age range of adolescence and young adulthood the body has a relatively high vitamin A requirement, so that the intake level of 1.6 I.U. per Calorie of food affords a smaller margin than in the earlier and later age periods.

Consistently with this view, when the level of intake is again twice as high (3.2 I.U. per Calorie of food) the potential surplus is so abundant that even in the age period of relatively higher requirement the body receives enough from the food to permit an uninterrupted trend of increasing reserve storage. This bodily storage, while always higher on the higher level of intake, is not so large as to account quantitatively for the whole difference of vitamin A content of the food consumed. From table 2 it may be seen that of the arithmetically potential surplus furnished by the food consumed, only about one-tenth to three-tenths was recovered as increase of the liver store.

With the moderate surpluses furnished by the experimental diets here used, the larger intake in food led to larger storage in the body both in absolute amount and when reckoned as percentage of the potential amount ingested. Of very much larger intakes only smaller percentages are stored; but these small percentages may mean large numbers of units, or bodily stores sufficient to meet the body's nutritional needs for a relatively long time (Bessey and Wolbach, '38; Moore, '37; Pett, '39; Ralli, Papper, and Baumann, '41; Sherman and Cammack, '26.)

For both the levels of intake here discussed it appears from the data in table 2, that bodily storage was most efficient in the earliest period of liberal vitamin A diet and later settled down to the storage of a lesser proportion as the storage capacity approached saturation or equilibrium with intake.

The general order of magnitude of the storages of vitamin A per gram of liver here found in rats are comparable with those observed in human livers by Ralli and coworkers ('41).

In the rats that were kept throughout their natural lives on the same three diets compared in the present paper, it was observed that the higher intake levels, which result in higher levels of bodily storage of vitamin A result also in more favorable life histories (Sherman, Campbell, Udiljak, and Yarmolinsky, '45.)

SUMMARY

In experiments with rats, a diet furnishing 3 I.U. of vitamin A per gram of air-dry food (0.8 I.U. per Calorie) has supported growth and life histories that are within the normal range but has resulted in the storage of only negligible reserves in the liver.

Parallel animals on diets containing, respectively, twice and four times as much vitamin A, acquired larger stores (and higher with the higher intake level) both per gram of liver and in total amount.

At the highest of these levels the body store increased steadily throughout the age range of the experiment (28 to 300 days). At the intermediate level there was a slackening of the storage trend at about 90 days, as if requirement were greatest at about that age.

In no case were differences in storage as great as if all the theoretically potential surplus had been stored, the actual efficiency of storage being of the order of 10 to 30% of the theoretically available surplus ingested in the food.

LITERATURE CITED

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

BESSEY, O. A., AND S. B. WOLBACH 1938 Vitamin A physiology and pathology. J. Am. Med. Assn., vol. 110, p. 2072.

CALDWELL, A. B., G. MACLEOD AND H. C. SHERMAN 1945 Bodily storage of vitamin A in relation to diet and age, studied by means of the antimony trichloride reaction and a photoelectric colorimeter. J. Nutrition, vol. 30, p. 349.

KAO, H. C., AND H. C. SHERMAN 1940 Influence of nutritional intake upon concentration of vitamin A in body tissues. Proc. Soc. Exp. Biol. Med., vol. 45, p. 589.

LITTLE, R. W., A. W. THOMAS AND H. C. SHERMAN 1943 Spectro-photometric studies of the storage of vitamin A in the body. J. Biol. Chem., vol. 148, p. 441.

MOORE, T. 1937 The vitamin A reserves of the adult human being in health and disease. Biochem. J., vol. 31, p. 155.

PETT, L. B. 1939 Vitamin A deficiency: Its prevalence and importance as shown by a new test. J. Lab. Clin. Med., vol. 25, p. 149.

RALLI, E. P., E. PAPPER AND K. PALEY 1941 Vitamin A and carotene content of human liver in normal and diseased subjects. Arch. Int. Med., vol. 68, p. 102.

REVIEW 1943 The metabolism of vitamin A. Nutrition Rev., vol. 1, p. 300.

ROHRER, A. B., AND H. C. SHERMAN 1943 The bodily store of vitamin A as influenced by age and by food. J. Nutrition, vol. 25, p. 605.

SHERMAN, H. C., AND L. C. BOYNTON 1925 Quantitative experiments upon the occurrence and distribution of vitamin A in the body, and the influence of the food. J. Am. Chem. Soc., vol. 47, p. 1646.

SHERMAN, H. C., AND M. L. CAMMACK 1926 A quantitative study of the storage of vitamin A. J. Biol. Chem., vol. 68, p. 69.

SHERMAN, H. C., H. L. CAMPBELL, M. UDILJAK AND H. YARMOLINSKY 1945 Vitamin A in relation to aging and to length of life. Proc. Natl. Acad. Sci., vol. 31, p. 107.

SHERMAN, H. C., AND E. N. TODHUNTER 1934 The determination of vitamin A values by a method of single feedings. J. Nutrition, vol. 8, p. 347.

BODILY STORAGE OF VITAMIN A IN RELATION TO DIET AND AGE STUDIED BY MEANS OF THE ANTIMONY TRICHLORIDE REACTION USING A PHOTO-ELECTRIC COLORIMETER [1]

ANNE B. CALDWELL, GRACE MACLEOD AND H. C. SHERMAN
Department of Chemistry, Columbia University, New York

(Received for publication June 27, 1945)

The earlier observations and experiments upon the importance of bodily storage as a factor in the nutritional significance of vitamin A were reviewed by Sherman and Cammack ('26) who studied quantitatively the effects of different levels and durations of preparatory feeding upon the storage of vitamin A in the body as judged by the survival period after transfer to vitamin A-free diet. In their experiments (with rats) they found, that the bodily store of vitamin A was promptly and markedly increased by the addition of cod liver oil to an already good diet but that the body only gradually approaches the attainment of its maximum store.

Subsequent development of methods for direct assay of the vitamin A content of tissues, and the finding of Baumann, Riising, and Steenbock ('34) that the assay of liver tissue alone is sufficient to indicate differences in the body store of this vitamin in well nourished animals, have facilitated further studies in this field. Lewis, Bodansky, Falk, and McGuire ('42) have compared the concentration of vitamin A in the blood with the storage in the liver as measured by the antimony trichloride colorimetric method. Using a spectrophotometric technique, Little, Thomas, and Sherman ('43) compared the concentrations of vitamin A in the livers of rats that had been fed diets of three levels of vitamin A value up to ages of 30, 90, and 150 days; while Rohrer and Sherman ('43), using the Todhunter method of assaying by a single-feeding technique, compared rats from three diets at the ages of 30 and 60 days. These last-mentioned investigations confirmed the importance of the level of intake but also indicated the desirability of more comprehensive studies to differentiate more clearly and conclusively

[1] Aided by grants from the Carnegie Institution of Washington and The Nutrition Foundation, Inc.

the respective relations of bodily storage and nutritional need at different ages as well as at different levels of intake.

The present paper reports the results of one series of such studies making use of an adaptation of the antimony trichloride method of assaying liver tissue for its vitamin A content. A simultaneous series in which the Todhunter method was employed is being reported separately.

EXPERIMENTAL

The experimental animals were rats of known hereditary and nutritional background, all of the same (Osborne-Mendel albino) strain, inbred in this laboratory through more than twenty generations. Different groups of these animals were fed in parallel on the following three diets: (1) Diet 16, consisting of five-sixths ground whole wheat and one-sixth dried whole milk, with 2% as much sodium chloride as wheat, the air-dry food mixture having a vitamin A value of 3 International Units per gram; (2) Diet 360, the same except for the addition of enough high-grade cod liver oil to bring the vitamin A value to 6 I.U. per gram; (3) Diet 361, the same with additional cod liver oil to bring the vitamin A value of this diet to 12 I.U. per gram. The amount of cod liver oil needed to thus fortify diets 360 and 361 was too small to affect significantly the composition and energy value of the diet or the intake of water-soluble vitamins. The food mixture and distilled water were available to each animal at all times.

Healthy, typical offspring of families that were in at least the second generation of their respective diets were killed at the ages of 30, 60, 90, 150, and 300 days and the liver tissue assayed by the antimony trichloride method as modified by Dann and Evelyn ('38) and by Lewis et al. ('42), using a photoelectric colorimeter (Coleman spectrophotometer) to measure the intensity of the transmitted light.

Table 1 shows for males and females, the average vitamin A content per gram of liver tissue at each of the five stated ages for each of the three diets, together with the probable errors of the means, the coefficients of variation, and the number of cases. While the females tend to store slightly higher concentrations of vitamin A than males of the same age on the same diet, the relationships of storage to level of intake and to age are essentially similar for the two sexes.

Table 2 shows correspondingly the averages for the same animals given in terms of International Units in the total liver. The larger size of the males (and their livers) approximately balances the higher concentration of vitamin A in the livers of the females.

TABLE 1

Vitamin A content per gram of liver tissue in rats.

AGE	DIET 16 (WITH 3 I.U./GM.)		DIET 360 (WITH 6 I.U./GM.)		DIET 361 (WITH 12 I.U./GM.)	
	Males	Females	Males	Females	Males	Females
30 days						
Mean ± its P.E.[1]	3. ± 1.0	2. ± 0.3	34. ± 2.1	31. ± 1.5	69. ± 3.0	87. ± 3.2
C.V.[2]	146	77	29	23	20	17
Number of cases	9	11	10	10	10	10
60 days						
Mean ± its P.E.	1. ± 0.2	2. ± 0.2	68. ± 3.2	73. ± 3.4	163. ± 5.3	205. ± 8.6
C.V.	93	63	27	22	15	20
Number of cases	16	12	15	10	10	10
90 days						
Mean ± its P.E.	1. ± 0.1	2. ± 0.1	51. ± 2.3	90. ± 5.0	244. ± 12.1	335. ± 14.2
C.V.	105	31	21	26	23	20
Number of cases	11	10	10	10	10	10
150 days						
Mean ± its P.E.	2. ± 0.1	10. ± 4.1	129. ± 5.7	193. ± 7.2	525. ± 27.9	680. ± 49.5
C.V.	47	240	21	18	25	34
Number of cases	11	14	10	11	10	10
300 days						
Mean ± its P.E.	0.1 ± 0.07	10. ± 2.9	226. ± 15.4	291. ± 20.8	1193. ± 41.0	1547. ± 32.7
C.V.	400	168	30	42	18	9
Number of cases	16	14	9	16	13	9

[1] Probable error. [2] Coefficient of variation.

TABLE 2

Vitamin A contents of total livers of rats: International Units.

AT THE AGE OF	DIET 16 (with 3 I.U./gm.)		DIET 360 (with 6 I.U./gm.)		DIET 361 (with 12 I.U./gm.)	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*
30 days	7	3	73	68	159	205
60 days	4	6	445	425	1165	1111
90 days	4	10	388	525	1905	2110
150 days	12	57	1162	1190	4424	3776
300 days	1	65	2167	1862	11453	9546

DISCUSSION OF RESULTS

Inasmuch as the rats were all of the same strain and each from a family that had been for at least a generation on the same diet as the respective experimental animal, it is noteworthy that even at the age of 30 days (end of infancy in the rat) the effects of the family diet are already significant in the concentrations and amounts of liver vitamin A.

On the diet with 3 I.U. of vitamin A value per gram of air-dry food or about 0.8 I.U. per Calorie the amount of storage of vitamin A in the liver at any of the five age levels investigated was so small as probably to be only negligible, though there might be considered to be a slight upward trend with maturity in the females but not in the males.

On the diet twice as rich in vitamin A value, but otherwise unchanged, both males and females increased the concentrations and total amounts of vitamin A in their livers in a fairly regular manner from the age of 30 to that of 300 days.

With the vitamin A value of the diet again doubled both males and females show increased rates and amounts of storage of vitamin A.

It is clear that when the vitamin A value of the food is high enough to furnish a surplus for storage in the liver, the amount stored and the concentration reached, are very significantly influenced both by the level of intake of the vitamin and the length of time that the body has been in daily receipt of a surplus.

The amounts of vitamin A here found agree with those obtained by the single-feeding method as reported in the accompanying paper (Campbell, Udiljak, Yarmolinsky, and Sherman '45) except that at the highest level of vitamin A intake and the two highest ages of the range studied, higher results were found by the method here used. Whether the rats, on this high level of vitamin A intake, changed some of the vitamin A to another form in the liver, which was inactive biologically but determined chemically, is in question.

SUMMARY

The effect of three levels of vitamin A on liver storage of this vitamin in the albino rat at 30, 60, 90, 150, and 300 days of age has been studied.

There was a statistically significant increase in the storage of vitamin A per gram of liver tissue as the level of intake was increased from 3 to 6 I.U. and from 6 to 12 I.U. per gram of diet.

At a level of intake of 3 I.U. per gram, there was very little storage of the vitamin and no increase in storage with increasing age up to 300

days. The vitamin A value of 3 I.U. per gram is not optimal and in the light of these storage results appears to be near the minimal limit of adequacy.

LITERATURE CITED

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

CAMPBELL, H. L., M. UDILJAK, H. YARMOLINSKY AND H. C. SHERMAN 1945 Bodily storage of vitamin A in relation to diet and age, studied by the assay method of single feedings. J. Nutrition, vol. 30, p. 343.

DANN, W. J., AND K. A. EVELYN 1938 CXXXVI. The determination of vitamin A with the photoelectric colorimeter. Biochem. J., vol. 32, p. 1008.

LEWIS, J. M., O. BODANSKY, K. G. FALK AND G. MCGUIRE 1942 Vitamin A requirements in the rat. The relation of vitamin A intake to growth and to concentration of vitamin A in the blood plasma, liver and retina. J. Nutrition, vol. 23, p. 351.

LITTLE, R. W., A. W. THOMAS AND H. C. SHERMAN 1943 Spectrophotometric studies of the storage of vitamin A in the body. J. Biol. Chem., vol. 148, p. 441.

ROHRER, A. B., AND H. C. SHERMAN 1943 The bodily storage of vitamin A as influenced by age and by food. J. Nutrition, vol. 25, p. 605.

SHERMAN, H. C., AND M. L. CAMMACK 1926 A quantitative study of the storage of vitamin A. J. Biol. Chem., vol. 68, p. 69.

THE AVAILABILITY OF ASCORBIC ACID IN PAPAYAS AND GUAVAS [1]

EVA R. HARTZLER
Nutritional Department, Hawaii Agricultural Experiment Station, Honolulu, Hawaii

TWO FIGURES

(Received for publication March 22, 1945)

Papayas and guavas are unusually rich sources of ascorbic acid (Miller et al., '44) surpassing even the citrus fruits. Since large amounts of these fruits are available in the Hawaiian Islands and elsewhere it is possible for them to make a significant contribution toward providing one of the essentials of an adequate diet. There is no information, however, on the availability to human beings of the ascorbic acid of these fruits. The experiments to be described in this paper were carried out in order to provide this information.

The plan of experiment was similar to that used by Clayton and Folsom ('40), Todhunter and Fatzer ('40) and by Clayton and Borden ('43). The data obtained by these workers indicated in general that the ascorbic acid provided by the foods studied was just as available to the body as was ascorbic acid taken in the pure form. The general principle of this method is to compare the excretion of ascorbic acid by subjects on an ascorbic acid-low diet: (a) when they are receiving the test foods; and (b) when they are receiving an equivalent amount of synthetic ascorbic acid.

Various workers refer to the difference between the amount of ascorbic acid ingested and that excreted as the amount utilized, but such a statement assumes that the ascorbic acid is completely available. Because of the possibilities of incomplete absorption or partial destruction in the intestinal tract this assumption is not justified. It has been clearly demonstrated, however, that the amount of ascorbic acid excreted in the urine increases when the amount ingested increases. From metabolism studies such as reported here, therefore, one may conclude that if equal excretions of ascorbic acid result when different sources of ascorbic acid are ingested, then the availability of the ascorbic acid to the body

[1] Published by permission of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 125.

must be equal. Furthermore this is true only if all other experimental conditions which might influence the excretion of ascorbic acid, such as the state of saturation of the body tissues, are kept constant. This is the assumption made in the present study in which two series of experiments involving fourteen different subjects were carried out.

METHODS

The diet. A balanced and varied diet [2] providing an average of less than 5.0 mg. of ascorbic acid per day was fed. The intake of foods containing small amounts of ascorbic acid was carefully controlled, but the subjects were allowed as much as they wanted of all other foods. Canned fruits, vegetables and evaporated milk were purchased in case lots and samples were assayed for vitamin C by dye titration. Fruit and vegetable juices were discarded and the vegetables were reheated in relatively large amounts of water which were also discarded. Fruits and vegetables used were canned apples, pears, peaches, figs, sweet corn, green beans, carrots, and beets; dried prunes, raisins, dates and figs; and raw apples without the skins. Since the diet was low in protective foods additional vitamins were provided in the form of capsulated preparations.

The papayas (Carica papaya — solo variety) weighed about one pound each and were grown on the Station farm at Poamoho. The guava juice was prepared according to the method of Miller et al. ('37) from common guavas (Psidium guajava) growing wild on the island of Oahu. The ascorbic acid content of the papayas ranged from 60 to 100 mg. per 100 gm. and that of the guava juice (a watery extract of cooked guavas) from 60 to 90 mg. per 100 ml.

Collection of samples. Twenty-four-hour urine samples were collected in brown glass bottles containing toluene and enough concentrated oxalic acid to bring the acid concentration of the final volume to 0.5%. Creatinine was determined daily on each sample as a check on the accuracy of the collections.

Ascorbic acid determinations. All ascorbic acid determinations were made by dye titration with the exception of plasma samples and foods yielding colored extracts which were assayed with the aid of a photoelectric colorimeter. The test foods were assayed each day. Individual papayas were assayed by taking at least four thin lengthwise slices as a composite sample. The individual portions given to the subjects were also in the form of lengthwise slices.

[2] Detailed records are on file in the office of the Nutrition Department of the Experiment Station and further information concerning the diet will be supplied upon request.

PLAN OF EXPERIMENTS

Experiment I. Nine subjects (4 females and 5 males, 19 to 33 years of age) serving from 3 to 12 weeks each were used in this experiment. Each subject was given 300 mg. of ascorbic acid per day for 3 days before starting the experiment. The experimental periods were as follows:

Saturation period — 1 day. Sunday. Regular diet at home plus 300 mg. ascorbic acid in forenoon.

Control period — 6 days. Monday through Saturday. Basal diet plus 75 mg. synthetic ascorbic acid per day.

Experimental periods — 6 days. Monday through Saturday. Basal diet plus one of the test foods in an amount providing 75 mg. of ascorbic acid per day.

All of the subjects except one repeated the control period at least twice. Two subjects received synthetic ascorbic acid in amounts other than 75 mg. per day for one or more periods in order to estimate the sensitivity of the method. All supplements were taken at 5:00 P. M., just before the evening meal except on Saturdays when they were taken at 12:00 noon.

Experiment II. Six subjects (3 females and 3 males, 29 to 34 years of age), one of whom had also served in experiment I, took part in this experiment. Each subject took 100 mg. of synthetic ascorbic acid per day for 4 days preceding the experiment. The subjects remained on the basal diet plus a constant intake of 75 mg. of ascorbic acid per day without interruption for 5 weeks. The supplements given during the 6 weeks of the experiment were as follows:

Preliminary adjustment period:

Week 1 — 6 days. 75 mg. synthetic ascorbic acid per day.
Week 2 — 7 days. 75 mg. synthetic ascorbic acid per day.

Experimental periods:

Week 3 — 7 days. Papaya equivalent to 75 mg. ascorbic acid per day.
Week 4 — 7 days. 75 mg. synthetic ascorbic acid per day.
Week 5 — 7 days. Guava juice equivalent to 75 mg. ascorbic acid per day.

The subjects in series II received their supplements at 5:00 P. M. each day throughout the experiment. Blood samples were taken on the afternoon of the sixth day of each period.

RESULTS AND DISCUSSION

Experiment I. The daily excretion of ascorbic acid for this group varied greatly due largely to the plan of experiment used. The day-to-day variations in excretion of ascorbic acid are illustrated in figure 1 which presents the individual 24-hour excretion values for subject D. On Mondays (first day of each period) the excretion values were generally quite high due to the high intake of the previous day. They then decreased throughout the week but frequently rose slightly on Saturday due to the change in time of administration of the supplement. The average excretions for each period may be compared, however, since

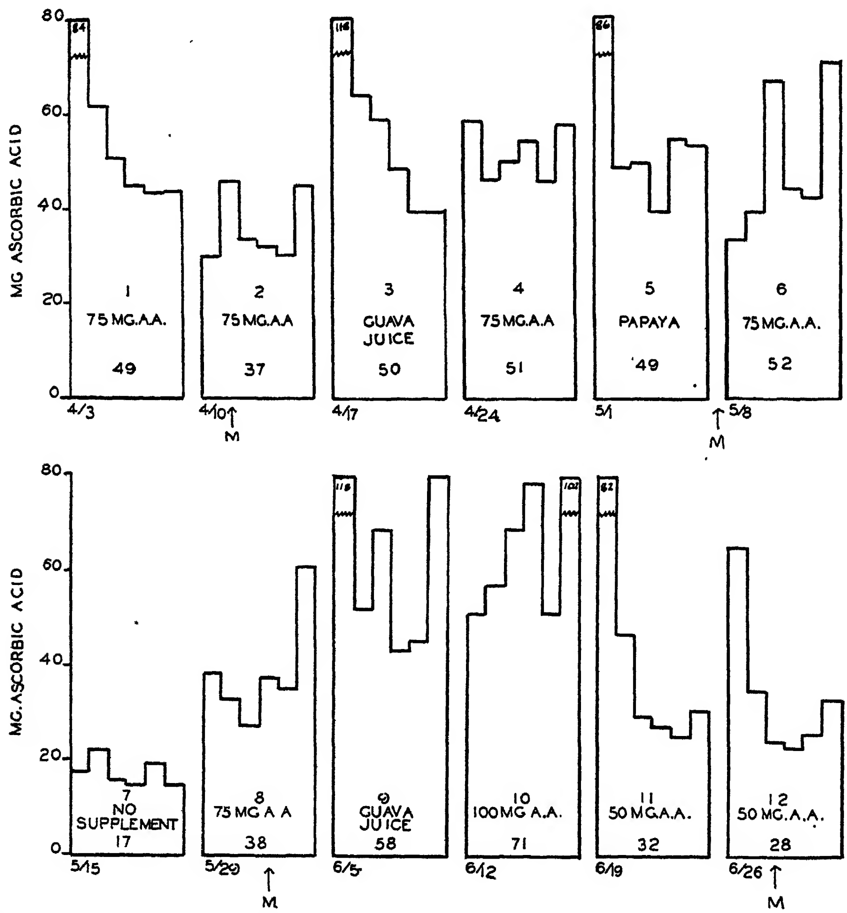


Fig. 1 The excretion of ascorbic acid by subject D during twelve supplemental periods. Each block represents on period of 6 days. The supplements given and the average excretion value for each period are written in the base of each block. Each bar represents the milligrams of ascorbic acid excreted in 24 hours. Broken bars represent excretions greater than 80 mg. The arrow and letter M indicate the first day of menstruation.

each period followed the same plan and each subject acted as his own control.

Table 1 shows the average excretion of ascorbic acid per period for each subject. The excretion values for the first day of each week were so extremely variable that they were omitted from the averages. The values given in the table, therefore, represent the average daily excretion for 5 days only.

Considerable variation occurred within subjects as well as between subjects. Two subjects, F and G, showed very little variation among

TABLE 1

Comparison of the excretion (5-day averages) of ascorbic acid by subjects of experiment I when receiving 75 mg. of ascorbic acid per day in the form of synthetic ascorbic acid, papayas or guava juice.

SUBJECT	AVERAGE EXCRETION PER PERIOD				DIFFERENCE		
	Control periods		Experimental periods		Maximum between control periods	From control periods	
	Individual periods	Average	Papaya	Guava juice		Papaya	Guava juice
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A	51 61	56	65	64	10	+9	+8
B	57 63	60	59	55	6	−1	−5
C	43 52 49	48	53	41	9	+5	−7
D	49 51 52	51	49	50 58	3	−2	−1 +7
E	45 44 50	46	53	40	6	+7	−6
F	43 44	44	45	44	1	+1	0
G	31	31	29	29	..	−2	−2
H	48 50	49	42	40	2	−7	−9
J	35 18 20	24	32	28	17	+8	+4
Av.		45	47	45	5	5	5

their average excretion values, but in the case of certain other subjects, (A, C, H, and J) the differences were large. The reproducibility of the results can be judged by noting the range of values for the control periods of a single subject.

An estimation of the significance of the differences between supplements can be made by comparing the values shown in columns 7 and 8 with those in column 6 of table 1. Column 7 which compares the excretions on papayas with the average excretions on synthetic ascorbic acid contains values ranging from 1 to 9. The values in column 8 which compares guava juice with synthetic ascorbic acid range from 0 to 9. Since these differences were no greater than those between individual control periods (1 to 10) they cannot be considered to be significant. It may be concluded, therefore, that within the limits of error of the method the ascorbic acid of guava juice and papayas was just as available as synthetic ascorbic acid.

In order to estimate the sensitivity of the method, subjects D and G served one or more periods in which they received 50 or 100 mg. of ascorbic acid per day, a variation of ± 30% from the usual control level. The differences between the excretions at these levels and the excretions at the 75-mg. level ranged from 19 to 38 mg. These differences were much greater than those between periods at the 75-mg. level and indicate that differences of ± 30% in the availability of ascorbic acid could readily be detected by the method used.

Experiment II. In this experiment the subjects after being maintained on a relatively high intake of ascorbic acid for 4 days were then held on a constant intake of 75 mg. per day (plus the small amount in the basal diet) throughout the 5 weeks of the experiment. This plan was adopted in an effort to reduce the daily fluctuations in the quantity of ascorbic acid excreted and thus to obtain data of greater significance. The individual 24-hour excretion values for all subjects of this experiment are shown in figure 2. Inspection of this figure reveals that all subjects excreted much more ascorbic acid during the first week than in later periods and in most cases a relatively constant level was not reached until after 2 weeks. For this reason the first 2 weeks are considered to be an adjustment period and only the last 3 weeks were used for the purpose of this experiment.

Fig. 2 The daily 24-hour excretions of ascorbic acid by subjects on experiment II. The figures inside the blocks show the average excretion for the period. The numbers 1 to 5 along the ordinate indicate weeks and supplements as follows: 1 and 2 — Adjustment period — 75 mg. synthetic ascorbic acid; 3, 4 and 5 — Experimental period — 3 — Papayas, 4 — 75 mg. synthetic ascorbic acid, 5 — Guava juice. The arrow and letter M indicate the first day of menstruation. D, K, and L — females; M, N, and O — males.

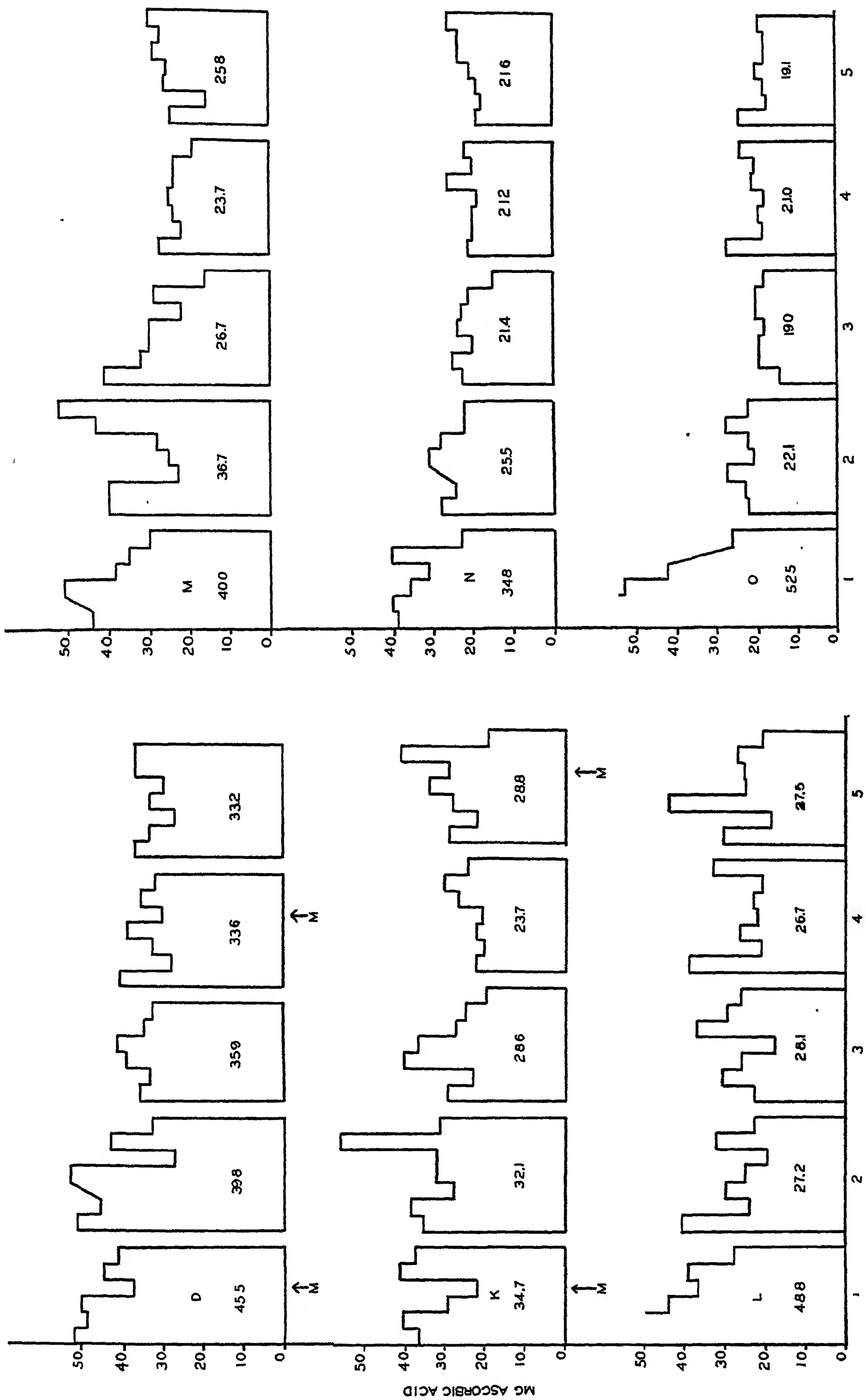


Figure 2

All the subjects showed some day-to-day fluctuation throughout the experimental periods but the men, particularly O and N, showed much less fluctuation than the women subjects. In general, however, a day with a high excretion was followed by a day with a low excretion and the weekly averages were remarkably constant. The averages for all six subjects for the 5 consecutive weeks were as follows: 42.7, 30.6, 26.6, 25.4 and 26.0 mg.

By subjecting the excretion data of the 5 weeks (fig. 2) to analysis of variance it was shown that there was no significant difference between the experimental weeks 3 — Papaya, 4 — Control, and 5 — Guava Juice. It may safely be concluded, therefore, that there was no significant difference in the availability of the ascorbic acid whether it was provided in the form of the pure compound or in the form of papayas or guava juice. There was a highly significant difference between weeks 1 and 2

TABLE 2

The ascorbic acid level of the plasma of subjects in experiment II.

SUPPLEMENT	MILLIGRAM PER CENT ASCORBIC ACID					
	D	K	L	M	N	O
Ascorbic acid	1.3	1.3	1.3	1.0	1.4	1.3
Ascorbic acid	1.2	1.3	1.3	0.7	1.3	1.0
Papaya	0.9	1.1	1.1	0.7	0.9	0.9
Ascorbic acid	1.0	0.9	1.0	0.7	0.9	0.7
Guava juice	1.1	1.1	0.9	0.9	0.9	0.9

(the adjustment period) and the chances were greater than 19 to 1 that the difference between the last week of the adjustment period and the first week of the experimental period was significant. The results of the statistical treatment, therefore, justify the omission of the data for the first 2 weeks from the final comparison of results.

The weekly plasma ascorbic acid values in table 2 confirm the conclusions drawn from the urinary excretion data. Slightly higher values were obtained during the first 2 weeks and only small differences occurred thereafter. All six subjects maintained very satisfactory plasma levels on an intake of approximately 80 mg. of ascorbic acid per day over a period of 5 weeks. The recommended allowance of the National Research Council is 75 mg. of ascorbic acid per day. The results obtained in this study indicate that the allowance is adequate to maintain normal plasma levels of ascorbic acid.

Effect of acidity or alkalinity. Various workers, Hawley et al. ('37), Goddard and Preston ('38) and Meyer and Hathaway ('44), have attempted to determine the effect of acidity or alkalinity upon the excretion of ascorbic acid, but their results so far have not been conclusive. No attempt was made in our experiments to study this factor and it was not rigidly controlled although the basal diet did not vary greatly and the subjects were asked not to take any alkalizing medicines. Any effect which may have occurred was small since the fruit supplements in the amounts used would have changed the acidity of the urine very little. This was demonstrated by an experiment to be reported elsewhere in which the ingestion for 3 days of amounts of papaya and guava juice five to ten times those given in this experiment caused only small changes in the pH of the urine.

Effect of menstruation. Early in the course of experiment I it was noted that very low values were obtained just before and/or during the early part of the menstrual period. From that time on care was taken to have the subjects receive synthetic ascorbic acid during these periods so that they could be compared with the control periods. The excretion values during these periods, in cases where they were well below the average for the individual, were omitted from the comparisons made in table 1.

Figure 1 which shows the daily excretion values of subject D for 12 weeks, including four menstrual periods, illustrates the effect of menstruation. The depressing effect of menstruation was pronounced in the second week as indicated by the average value of 37 mg. as compared with the normal value for this subject of 51 mg. Also the value for the first day of the week, which ordinarily was very high due to the saturation period of the preceding day, was very low — 30 mg. The next menstrual period which occurred during the sixth week began on the day of the saturation period and again resulted in a low value on the first day of the week. The average value for week 6, however, was normal (52 mg.) since the value for Monday was not included in the 5-day average and several high values occurred later in the week. The third menstrual period in week 8 was very similar to that in week 2 with a low value on the first day and a low average of 38 mg. for the week. During weeks 1, 2, 3, 4, 5, 6, 8 and 9, in all of which the subject was receiving 75 mg. of ascorbic acid per day in one form or another, no daily values below 35 mg. occurred except just preceding or during menstrual periods. During these menstrual periods there were seven daily values below 35 mg., two of which occurred on Mondays. The values for the 5 Mondays of normal weeks ranged from 58 to 118 with

an average of 93 mg. of ascorbic acid while those which occurred in the 3 weeks of menstruation ranged from 30 to 38 with an average of 34 mg. ascorbic acid. Because of the magnitude of these differences it is not reasonable to suppose that they occurred by chance.

In experiment II, however, little or no effect of menstruation was noted. No satisfactory explanation is apparent for this discrepancy between the observations made in the two experiments. In experiment II the concentration of ascorbic acid in the tissues was undoubtedly lower than in experiment I because of the lack of weekly 1-day saturation periods, but otherwise the conditions were similar. Subject L in this group was in the first month of pregnancy so menstruation was not a factor, but this subject showed more fluctuation than any other.

No mention of any effect of menstruation on the excretion of ascorbic acid has been made by other workers studying the availability of ascorbic acid. Pillay ('40), however, observed a relation between ovulation time and minimum excretion of ascorbic acid and Mickelsen et al. ('43) noted an increase in the plasma ascorbic acid level of women during the middle of the menstrual cycle. From the data accumulated in this study it would appear that there is some relation between menstruation and ascorbic acid excretion. It may be of little significance from the viewpoint of the nutritionist, but it is a factor to be considered in carrying out ascorbic acid metabolism studies.

SUMMARY AND CONCLUSIONS

The availability of the ascorbic acid of papayas and guava juice was determined by comparing the urinary excretion of ascorbic acid by human subjects maintained on a diet low in ascorbic acid: (a) when receiving 75 mg. of synthetic ascorbic acid per day; and (b), when receiving an equivalent amount of ascorbic acid in the form of papayas or guava juice. Two experiments involving a total of fourteen subjects (eight men and six women) were carried out.

The two experiments differed from each other only in the way in which the experimental periods were divided. In the first experiment 6-day periods preceded by a 1-day saturation period were used. In the second experiment the subjects remained continuously on the basal diet plus 75 mg. of ascorbic acid for 5 weeks, the first 2 weeks serving as a preliminary adjustment period and only the last 3 weeks being used for purposes of comparison. This latter procedure greatly reduced the variation in the data obtained.

No significant differences in the availability of the ascorbic acid of papayas or guava juice as compared with synthetic ascorbic acid were found in either experiment.

Blood plasma ascorbic acid levels which were determined in experiment II were similar in all experimental periods. Subjects receiving 75 to 80 mg. of ascorbic acid per day over a period of 5 weeks maintained plasma levels of from 0.7 to 1.0 mg. %.

In some instances menstruation appeared to have a depressing effect on the excretion of ascorbic acid.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to Prof. Carey D. Miller, Head of the Nutrition Department, who suggested the problem and made many helpful suggestions, to Dr. E. L. Willett of the Division of Animal Husbandry who carried out the analysis of variance, to Miss Winifred Ross, Junior Nutritionist, who planned and supervised the preparation of meals in experiment II and Dr. E. A. Fennel and staff at the Clinic of Physicians and Surgeons who took the blood samples. The author also wishes especially to thank the various members of the Experiment Station Staff who served as subjects for these studies.

LITERATURE CITED

CLAYTON, MARY M., AND RUBY A. BORDEN 1943 The availability for human nutrition of the vitamin C in raw cabbage and home-canned tomato juice. J. Nutrition, vol. 25, p. 349.

CLAYTON, MARY M., AND MARIE T. FOLSOM 1940 A method for the study of the availability for human nutrition of the vitamin C in foods, with an application to the study of the potato. J. Home Econ., vol. 32, p. 390.

GODDARD, VERZ ROGERS, AND RUTH ALICE PRESTON 1938 Effect of diet on vitamin C output. J. Home Econ., vol. 30, p. 482.

HAWLEY, ESTELLE E., RAY G. DAGGS AND DORAN J. STEPHENS 1937 The effect of the administration of acid and alkaline salts upon the ascorbic acid content of guinea pig tissues. J. Nutrition, vol. 14, p. 1.

MEYER, FRIEDA L., AND MILICENT L. HATHAWAY 1944 Further studies on the vitamin C metabolism of preschool children. J. Nutrition, vol. 28, p. 93.

MICKELSEN, O., A. L. DIPPEL AND R. L. TODD 1943 Plasma vitamin C levels in women during the menstrual cycle. J. Clin. Endocrinol., vol. 3, p. 600.

MILLER, CAREY D., KATHERINE BAZORE AND RUTH C. ROBBINS 1937 Some fruits of Hawaii. Their composition, nutritive value and use. Bulletin no. 77, Hawaii Agricultural Experment Station, Honolulu, Hawaii.

MILLER, CAREY D., LUCILLE LOUIS AND KISAKO YANAZAWA 1944 Vitamin Values of Hawaiian Grown Fruits and Vegetables. Progress Notes. No. 36. Hawaii Agricultural Experiment Station. University of Hawaii.

PILLAY, A. P. 1940 Vitamin C and Ovulation. Indian Med. Gaz., vol. 75, p. 91.

TODHUNTER, E. N., AND ALVA S. FATZER 1940 A comparison of the utilization by college women of equivalent amounts of ascorbic acid (vitamin C) in red raspberries and in crystalline form. J. Nutrition, vol. 19, p. 121.

NUTRITIONAL STUDIES WITH THE DUCK

II. PYRIDOXINE DEFICIENCY [1]

D. M. HEGSTED AND M. N. RAO [2]

Department of Nutrition, Harvard School of Public Health, and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

ONE FIGURE

(Received for publication July 20, 1945)

Pyridoxine deficiency has been studied quite extensively in four species: rats, dogs, pigs, and chicks. The deficiency symptoms apparently common to these species (Lepkovsky, '42) are lack of growth, anemia, and convulsions or fits. Convulsions, however, are not consistently noted in all laboratories (Briggs et al., '42), and the anemia is apparently mild or variable in rats and chicks (Fouts and Lepkovsky, '42; Kornberg, Tabor and Sebrell, '45; Luckey et al., '45). Acrodynia is observed in deficient rats but not in the other species. A disturbance in tryptophane metabolism resulting in the excretion of large amounts of xanthurenic acid was first observed in rats by Lepkovsky (Lepkovsky and Nielson, '42; Lepkovsky, Roboz and Haagen-Smit, '43) and has been noted and studied in swine by Cartwright et al. ('44). Deficient dogs, however, excrete very little xanthurenic acid, and none was noted in the droppings of deficient chicks (Lepkovsky, Roboz and Haagen-Smit, '43). It thus appears that certain symptoms may be considered as characteristic of pyridoxine deficiency while others may at this time be considered as only species specific.

Pyridoxine deficiency is easily produced in young ducklings by the omission of pyridoxine from a purified ration previously described (Hegsted and Stare, '45). In this paper we wish to present the results of studies of this deficiency in this species.

EXPERIMENTAL

The pyridoxine deficient ration which was fed ad libitum had the following percentage composition: sucrose 55.7, casein (SMA vitamin-free) 18, gelatin 10, corn oil 4, salt mixture (Hegsted et al., '41) 5,

[1] Supported in part by a grant-in-aid from the John and Mary R. Markle Foundation.

[2] Rockefeller Fellow at the Harvard School of Public Health, 1943 to 1945.

mono-calcium phosphate 1, cod liver oil 2, liver extract [3] 4, and choline chloride 0.3. Crystalline vitamins [4] were added to supply the following amounts per 100 gm. of ration: thiamine 400 μg., riboflavin 800 μg., calcium pantothenate 1.5 mg., biotin 20 μg., and nicotinic acid 3.0 mg. Ten milligrams of alpha tocopherol and 10 μg. of menadione dissolved in corn oil were fed twice weekly by dropper. Control animals received the same diet plus 400 μg of pyridoxine hydrochloride per 100 gm. All of the birds were kept in heated cages with raised screen bottoms as previously described.

Acute deficiency in day-old ducklings

In experiment I the deficient diet was fed to 4-day-old white Pekin ducklings while four others received the same diet to which pyridoxine was added. The animals were weighed daily and observed for symptoms of deficiency. Hemoglobin and red cell counts were made at weekly intervals. Blood smears were also taken and stained supravitally with 0.2% alcoholic Azure II followed by Wright-Giemsa counter stain.

The weight curves and the hematological data are shown in figure 1. After the birds had received the deficient diet for about 3 weeks, one of the birds died. To keep the other birds alive, a small amount of pyridoxine (50 μg. per 100 gm. of ration) was added to the ration for a period of 4 days. The data show that on the ration lacking in pyridoxine the growth was very poor, and this was accompanied by the development of a severe anemia. Data obtained the day before the one duck died showed a hemoglobin value of 3.5% and a red cell count of 900,000 per cu. mm., while figures for the control birds were 7.35% hemoglobin and 1,810,000 red cells per cu. mm.[5] All three of the remaining birds showed a prompt response to this small amount of pyridoxine as shown by the growth curves and by the blood picture. It is apparent that at the time the experiment was ended the birds were again showing anemia.

From the blood smears it was possible to compare the relative size of the cells from the deficient and control birds. The length and width of twenty-five cells in a smear from a deficient bird were measured with a calibrated micrometer eye-piece. They showed a mean maximum length of 11.04 μ, mean maximum width of 6.46 μ. Similar data from a control

[3] Fraction "L" supplied by The Wilson Laboratories, Chicago.

[4] Supplied by Merck and Co., Inc., Rahway, New Jersey.

[5] The following data are the average results obtained with young ducklings 4 to 10 days old which received commercial duck pellets: Hemoglobin — 8.19 gm./100 ml.; red blood cells — 1.98 million per cu. mm.; hematocrit — 28.8 ml./100 ml.; mean cell volume — 141 μ^3; mean cell hemoglobin — 41.8 $\mu\mu$g.; mean cell hemoglobin concentration — 29.4%.

duck were mean maximum length 11.45 μ, and mean maximum width 7.19 μ. Assuming that the cells are perfectly ellipsoidal, the areas were calculated according to the formula: Area $= \pi/4 \times$ length $\times$ width. This gave a mean area of 56.21 $\mu^2 \pm 8.42$ for the cells of the deficient bird compared with 64.67 $\mu^2 \pm 4.12$ for the control. The difference is 8.46 μ^2, a value 4.5 times the standard error of the difference. Thus the

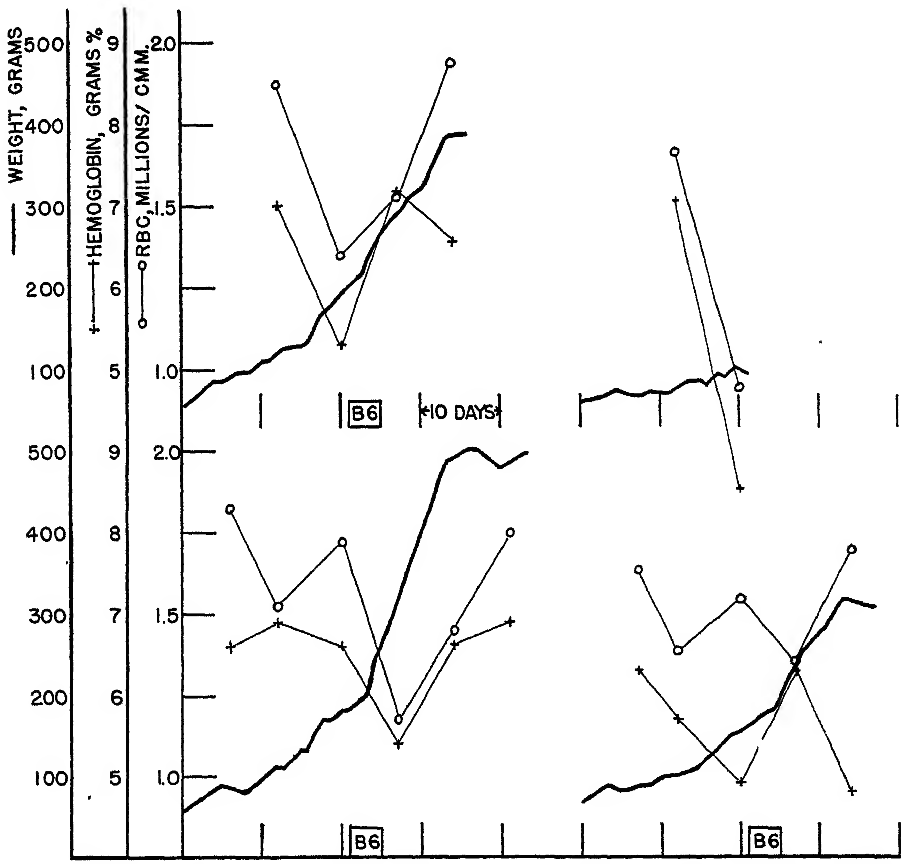


Fig. 1 Data showing the development of pyridoxine deficiency in day-old ducklings. Ration containing 50 μg. of added pyridoxine (per 100 gm.) was fed during the B_6 period.

cells from the deficient birds are significantly smaller than those from the controls. This is also borne out by studies on older ducks reported below.

No clear-cut symptoms other than poor growth and anemia were observed in the deficient birds. They were weak and small, but no dermatitis or nervous symptoms were seen although these were specifically looked for.

Requirements for growth

In an attempt to establish the approximate requirement for pyridoxine, five groups of four ducks each were fed the basal diet to which were added 0, 30, 50, and 100 µg. pyridoxine hydrochloride per 100 gm. of ration, respectively. In a second similar experiment, levels of 50, 75, and 100 µg. per 100 gm. were fed. The results obtained during a 2-week period in these experiments are summarized in table 1. It appears that the duck requires approximately 100 µg. of pyridoxine per 100 gm. of ration in addition to that already present in the basal diet. This basal diet is very similar to ration 483B used by Briggs et al. ('42) with chicks which was found to contain about 100 µg. per 100 gm. However,

TABLE 1

Studies on the pyridoxine requirement of the duckling.

PYRIDOXINE ADDED TO BASAL RATION	NUMBER OF DUCKS	GAIN PER DUCK PER DAY	GAINS AS PER CENT OF CONTROLS
µg./100 gm.		*gm.*	
None	7 [1]	6.9	25.6
30	4	11.6	43.0
50	7 [1]	17.3	64.0
75	4	23.2	86.0
100	7 [1]	26.3	97.5
400	7 [1]	27.0	100.0

[1] Average of two experiments, one group of 4 and another of 3 animals.

the liver extract we have used was not alcohol extracted. Assuming a figure of 100 to 150 µg. of pyridoxine per 100 gm. of basal ration, the requirement of the duck would be about 200 to 250 µg. per 100 gm. of ration. This value is similar to that found for chicks (Briggs et al., '42), 275 to 300 µg. per 100 gm. of ration. It is apparent that the requirements of the chick and the duck based upon the content of the ration are not greatly dissimilar.

Chronic pyridoxine deficiency

Pyridoxine deficiency in four ducks from 2 to 3 weeks old was produced by feeding the same ration. These birds had received duck pellets until they were started on the experimental regime. They showed a failure of growth soon after being placed on the deficient diet. Feathering was poor and practically all of the feathers were lost over the back and sides, and the pinion feathers did not develop. As noted earlier (Hegsted and Stare, '45), poor feather development on this ration may be related

to deficiencies of unidentified factors. A paralysis developed which was characterized by weakness and difficulty in standing. At times the birds sat on their hocks with the head laid on the floor. The toes had a tendency to turn in, and the birds apparently could not extend their toes to stand squarely on their feet. Occasional periods of violent tremors were observed during which the birds shook violently as if suffering from a chill. A few minutes later they might appear quite normal except for a slow uncertain gait or might be quite weak and fall forward on the breast and then be unable to pull their feet forward under the body. The whole picture was variable from day to day, severe symptoms occurring 1 day but relatively normal appearance other than the condition of the feathers a few hours or a day later.

Hemoglobin,[6] red cell counts, and hematocrit determinations were made at intervals during the development of the deficiency and after treatment with pyridoxine. The complete protocols of two birds are shown in table 2. Duck no. 1 was bled at intervals at the beginning in order to put some strain upon the hematopoietic organs and this bird developed the deficiency before the others. The blood picture is characterized by a drop in hemoglobin, red cell count, and hematocrit. The decrease in these three is, however, not proportional so that the anemia, as shown by the calculation of mean cell volume, is generally microcytic. Normal cell volumes of approximately 140 fall to levels as low as 85. There appears to be a slow development of the anemia which is followed by a crisis when the values drop precipitously. In some of the birds, the mean cell hemoglobin and the mean cell hemoglobin concentration fell to low levels. At these times the blood smear was characterized by the presence of large numbers of young red cells.

The response to pyridoxine is exceedingly prompt. The blood picture becomes approximately normal within a few days. A growth response occurs immediately as well.

DISCUSSION

Growth failure is the first symptom of pyridoxine deficiency noted in these animals. The duck is remarkable in the speed with which it develops vitamin deficiencies. Differences in the rate of gain are clearly evident within 3 or 4 days after day-old ducklings are given the deficient ration, in spite of the fact that the basal diet probably contains about

[6] Ten cu. mm. of blood were diluted with 10 ml. of N/10 HCl. After a few minutes standing, 1 ml. of 2N NaOH was added to dissolve the cell stroma after which the hemoglobin content was determined with a Coleman Spectrophotometer at 380 mμ. Readings were compared with a standard curve made from duck blood which had been analyzed for O_2 capacity by the Van Slyke method.

DUCK NUMBER 1

Date	Body weight	Hemoglobin	Red blood cells	Hematocrit	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Supplement
	grams	%	*millions per cubic millimeter*	*ml./100 ml.*	*cubic micra*	*micromicrograms*	%	
2/3	490							
2/7	550							
2/10 [1]	610							
2/14 [2]	610	11.0	2.56	37.5	146	41.4	29.4	
2/16 [2]	600	8.8						
2/20	615	6.9						
2/21 [2]		7.2	1.82	27.1	148	39.6	26.2	
3/1	530	9.8						
3/12	500	5.6	1.73	18.2	105	32.4	31.1	
3/13	480	3.5	1.10	10.0	100	31.7	35.0	
3/14								1 mg. B_6 oral
3/15	540	4.9	1.51	21.0	139	32.5	23.4	
3/30	690	10.4	2.94	34.5	118	35.0	29.8	
3/31	655							
4/2	705	9.5	2.81	32.5	115	33.8	29.2	
4/4	760	8.1	2.42	27.5	114	33.6	29.4	
4/6	695	9.6	2.45	31.0	126	39.2	31.0	
4/9	705	9.7						
4/18	740	8.5	2.09	26.0	124	40.6	32.6	
4/19		8.5						
4/20	745	8.0	2.34	26.0	111	34.2	30.8	
4/21								
4/23	720	7.5	2.58	26.5	99	29.0	28.3	
4/24								
4/25								
4/26	695	8.0	2.34	28.5	121	33.9	28.0	
4/27	655	7.0	2.27	25.0	115	30.8	28.0	
4/30	655	7.1	1.99	25.5	128	35.6	27.9	
5/4	755	6.4						
5/6	690	6.0	1.55	20.5	132	38.7	29.3	
5/9	615	4.7	1.42	18.5	130	33.1	25.4	
5/10		4.9		20.5				10 mg. B_6
5/11	755	6.9	1.63	24.0	147	42.2	28.8	
5/12	855	5.7	1.73	22.0	127	33.0	25.9	
5/15	980	8.8	2.25	31.0	137	39.1	28.4	

DUCK NUMBER 2

Date	Body weight	Hemoglobin	Red blood cells	Hematocrit	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Supplement
	grams	%	*millions per cubic millimeter*	*ml./100 ml.*	*cubic micra*	*micromicrograms*	%	
2/3	515							
2/7	580							
2/10 [1]	695							
2/14 [2]	695	10.1	2.52	34.0	135	40.0	29.8	
2/16 [2]	710	9.6						
2/20	800	8.7						
2/21 [2]		8.8	2.20	33.5	152	40.0	26.2	
3/1	795	9.4						
3/12	820							
3/13	800	8.2	2.37	32.0	135	34.6	26.6	
3/14								
3/15	814	9.6	2.56	36.5	143	37.4	26.3	
3/30	905	6.8	2.44	27.5	113	28.0	25.0	
3/31	880	7.2	2.50	26.5	106	28.8	27.2	
4/2	885	7.6	1.97	26.2	130	37.0	29.0	
4/4	870	6.8	1.78	24.0	135	38.2	28.4	
4/6	890	6.7	1.91	25.2	132	35.0	25.6	
4/9	930	7.1						
4/18	920	4.2	1.86	20.5	110	22.6	20.5	
4/19		4.4	1.88	21.0	112	23.4	21.0	
4/20	905	4.4						2 mg. B_6
4/21								
4/23	880	4.4	2.00	22.0	110	22.0	20.0	2 mg. B_6
4/24								
4/25		5.1	2.00	24.0	120	25.5	21.3	
4/26	995	7.3	1.90	27.5	145	38.4	26.6	
4/27	990	10.4	2.16	36.0	167	48.1	28.9	
4/30	1010	12.5	2.75	44.0	160	45.5	28.5	
5/4	1260	11.9						
5/6	1250							
5/9	1255							
5/10								
5/11	1150							
5/12		10.3						
5/15	1080	9.6						

[1] Started on experimental ration. [2] 15 ml. blood removed.

50% of the total pyridoxine requirement. This is no doubt related to the extremely rapid rate of gain which at some stages is equal to 25% of the body weight per day.

The anemia develops more slowly and paralysis and convulsions appear to be symptoms of chronic deficiency. Periods of convulsion were common in the older ducks which survived long periods upon the deficient ration but were not observed in very young ducklings. Thus some of the difference in species noted earlier may be simply differences in certain of the symptoms related to the age of the animals and relative degree of deficiency. Rations completely lacking in a vitamin may produce acute symptoms much different from those which develop over longer periods of time. This has been clearly shown by Shaw, Phillips and Elvehjem ('45) in vitamin C deficiency in the monkey. It is as yet uncertain whether a derangement of tryptophane metabolism occurs in birds (Lepkovsky, Roboz and Haagen-Smit, '43). Attempts to demonstrate xanthurenic acid in the droppings of severely deficient ducks were unsuccessful.

SUMMARY

Severe acute pyridoxine deficiency in young ducklings is characterized by growth failure accompanied by severe anemia. Neither convulsions nor paralysis were observed in these birds. The pyridoxine requirement of young ducklings is similar to that of chicks, approximately 250 μg. per 100 gm. of ration.

Chronic pyridoxine deficiency in older ducklings produced lack of growth, paralysis, convulsions, severe microcytic anemia, and poor feather development.

LITERATURE CITED

BRIGGS, G. M., JR., R. C. MILLS, D. M. HEGSTED, C. A. ELVEHJEM AND E. B. HART 1942 The vitamin B_6 requirement of the chick. Poultry Sci., vol. 21, p. 379.

CARTWRIGHT, G. E., M. M. WINTROBE, P. JONES, M. LAURITSEN AND S. HUMPHREYS 1944 Tryptophane derivatives in the urine of pyrdoxine-deficient swine. Bull. Johns Hopkins Hosp., vol. 104, p. 34.

FOUTS, P. J., AND S. LEPKOVSKY 1942 A green pigment-producing compound in urine of pyridoxine-deficient dogs. Proc. Soc. Exp. Biol. Med., vol. 50, p. 221.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., vol. 138, p. 459.

HEGSTED, D. M., AND F. J. STARE 1945 Nutritional studies with the duck. I. Purified rations for the duck. J. Nutrition, vol. 30, p. 37.

KORNBERG, A., H. TABOR AND W. H. SEBRELL 1945 Blood regeneration in pyridoxine-deficient rats. Am. J. Physiol., vol. 143, p. 434.

LEPKOVSKY, S. 1942 Pyridoxine. The Biological Action of the Vitamins. University of Chicago Press, pp. 111–119.

LEPKOVSKY, S.. AND E. NIELSON 1942 A green pigment-producing compound in urine of pyridoxine-deficient rats. J. Biol. Chem., vol. 144, p. 135.

LEPKOVSKY, S., E. ROBOZ AND A. J. HAAGEN-SMIT 1943 Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine-deficient rats. J. Biol. Chem., vol. 149, p. 195.

LUCKEY, T. D., G. M. BRIGGS, JR., C. A. ELVEHJEM AND E. B. HART 1945 Activity of pyridoxine derivatives in chick nutrition. Proc. Soc. Exp. Biol. Med., vol. 58, p. 340.
p. 340.

SHAW, J. H., P. H. PHILLIPS AND C. A. ELVEHJEM 1945 Acute and chronic ascorbic acid deficiencies in the Rhesus monkey. J. Nutrition, vol. 29, p. 365.

THE EFFECT OF GENETIC VARIATION IN THE FOWL ON THE THIAMINE CONTENT OF THE EGG [1]

NEVIN S. SCRIMSHAW,[2] F. B. HUTT [3] AND MARY W. SCRIMSHAW [2]

WITH THE ASSISTANCE OF C. R. SULLIVAN

Department of Physiology and Vital Economics, School of Medicine and Dentistry, The University of Rochester, Rochester, New York; and Department of Poultry Husbandry, Cornell University, Ithaca, New York

(Received for publication June 18, 1945)

Differences in the vitamin content of eggs and other food products are known to occur as the result of many factors. Of these none are more interesting than those variations which are based on genetic differences. The data presented here show that the thiamine content of the eggs of one of the common breeds of domestic fowl differs markedly from that in two others.

Some years ago, Nichita and Iftimesco ('34), and Nichita, Tuschak and Calcef ('34) found that adult White Leghorns could withstand a deficiency of vitamin B_1 much better than Rhode Island Reds. Since the diets used for the two breeds were not identical, there was some question as to whether these breeds differed genetically in nutritional requirements. Lamoreux and Hutt ('39), using chicks instead of adults, proved that White Leghorns as a breed are more resistant than either Rhode Island Reds or Barred Plymouth Rocks to a deficiency of vitamin B_1. Their experiments gave little clue to the basis for the difference and the present study was made to obtain further information on this point.

PROCEDURE

Whereas in the previous experiments the procedure was to determine how well the test animals could withstand diets deficient in thiamine, in the investigation here reported all birds were given a diet containing ample thiamine and the breeds were then compared with respect to the amount of that substance deposited in their eggs.

While the quantitative requirement of thiamine by mature laying fowls has not yet been determined, it is not likely to be greater than

[1] Aided by a Research Fellowship from Swift and Company, Chicago, Illinois. Number 20 in the series by F.B.H. entitled "Genetics of the Fowl."

[2] At the University of Rochester.

[3] At Cornell University.

that for growing chicks, which Arnold and Elvehjem ('38) found to be 60–80 µg. added per 100 gm. of "low thiamine" feed. Upon analysis, the thiamine content of the grain used in this experiment was found to be 410 µg., and of the mash 540 µg. per 100 gm. of feed. The birds' diet consisted of approximately half grain and half mash, and it is obvious that they consumed an abundance of thiamine.

The eggs analyzed for breed comparisons were laid at Cornell University by pullets varying in age from 7 to 9 months. The flocks of the different breeds were kept under comparable conditions and none of the birds had been laying for more than about 3 months. On December 19, 1944, twenty-four eggs, each laid by a different hen, were taken for analysis. Twelve of these were laid by White Leghorns and twelve by Rhode Island Reds. Two weeks later another twenty-four eggs were taken on 1 day. Of these, half were laid by twelve different Barred Rocks, and the rest by twelve White Leghorns, none of which had contributed to the previous sample. These eggs were all assayed individually, thus yielding determinations for twenty-three different White Leghorn pullets and for eleven of each of the two heavy breeds (one egg was lost through accident in each lot).

Other White Leghorn eggs from a poultry farm near Rochester were analyzed at intervals from January, 1944 to April, 1945. The grain fed this flock contained 400 µg. of thiamine and the mash 580 µg. per 100 gm. of feed. Although analysis was done only on the feed of April, 1945, the hens were presumably on the same diet during the entire period studied. Before analysis the eggs were weighed, broken, and the yolk carefully separated from the white by repeated pourings between the half shells. The white was weighed separately and the total weight obtained after the yolk was added. These egg contents were then mixed in a Waring Blender. Thirty grams of the blended contents were taken as a sample, diluted to 250 ml., acidified to pH 4.0 or below, and stored at about 5°C. All thiamine assays were carried out by the macro-fermentation method of Schultz et al. ('42) as modified by Scrimshaw and Stewart ('44). Nearly half of the samples were routinely checked by two separate assays, including any determinations that seemed to be out of range.

RESULTS

In table 1 the thiamine values are expressed as (1) the amount per egg, (2) the amount per 100 gm. of egg contents (to permit comparison with other determinations thus expressed), and (3) the amount per 100 gm. of yolk. This last figure is probably the best measure of thia-

TABLE 1

Assay of thiamine in eggs of different breeds.

HEN NO.	WEIGHT IN GRAMS: Whole egg	White	Yolk	THIAMINE IN MICROGRAMS PER: Egg	100 gm. egg contents	100 gm. yolk
		I. White Leghorns				
1	55.7	31.0	17.5	50.0	103	286
2	50.2	29.0	14.8	49.1	111	332
3	57.0	32.5	17.7	39.7	79	224
4	52.0	30.3	15.9	54.4	120	342
5	53.0	31.2	15.6	47.7	102	306
6	52.1	28.0	18.2	55.4	120	304
7	51.0	27.9	17.4	50.8	90	234
8	49.9	26.7	17.6	45.2	102	257
9	60.1	37.4	15.6	56.2	106	360
10	55.7	31.6	16.9	39.8	82	236
11	50.4	29.0	15.2	47.8	108	314
12	53.7	27.5	19.5	48.9	104	251
13	48.8	25.8	17.0	36.4	85	214
14	55.5	29.2	18.3	53.7	133	293
15	54.6	29.1	19.1	52.5	109	275
16	55.9	29.2	19.9	51.1	104	257
17	51.8	27.3	17.8	49.6	110	279
18	52.7	26.1	20.5	50.8	109	248
19	49.4	26.3	18.0	47.4	107	263
20	61.7	34.0	19.2	60.1	113	313
21	57.0	30.5	17.3	44.5	93	257
22	53.8	26.8	18.6	52.2	115	281
23	53.5	26.5	18.7	53.8	119	288
Mean	53.7	29.3	17.7	49.0	104.5	278.9
St. dev.			1.5	5.9	11.6	38.1

HEN NO.	WEIGHT IN GRAMS: Whole egg	White	Yolk	THIAMINE IN MICROGRAMS PER: Egg	100 gm. egg contents	100 gm. yolk
		II. Rhode Island Reds				
1	64.4	34.3	22.5	35.8	63	159
2	50.1	26.5	17.0	31.8	73	187
3	61.7	37.7	16.5	20.6	38	125
4	68.1	37.3	23.4	43.1	71	184
5	62.7	34.2	21.2	38.8	70	183
6	55.1			35.4	79	
7	63.6	36.3	20.5	36.4	64	178
8	62.4	31.8	23.5	38.2	69	163
9	62.0	36.0	18.0	27.0	50	150
10	58.1	32.6	18.5	31.2	61	169
11	61.8	33.0	22.3	38.7	70	174
Mean	60.9	34.0	20.2	34.3	64.4	167.2
St. dev.			2.7	6.3	11.5	19.0
		III. Barred Plymouth Rocks				
1	64.3	37.3	20.0	47.0	82	235
2	59.5	33.0	19.2	34.5	66	180
3	49.7	27.0	16.6	28.8	66	173
4	62.7	35.9	18.2	29.8	55	164
5	60.5	35.4	17.9	28.9	54	161
6	52.4	28.1	18.3	23.2	50	127
7	58.7	31.3	19.9	35.8	70	180
8	60.0	32.7	19.6	39.2	75	200
9	53.2	28.7	17.9	29.4	63	164
10	52.8	26.7	19.3	34.5	75	179
11	58.0	28.0	22.8	36.1	71	158
Mean	57.4	31.3	19.1	33.4	66.1	174.6
St. dev.			1.6	6.4	9.9	27.1

mine content for breed comparisons since all of the thiamine is originally deposited in the yolk (Chick and Roscoe, '29; Ellis et al., '33; Baker and Wright, '35; Bethke et al., '36; Scrimshaw and Stewart, '44). If the thiamine content is not described in this manner, consistent differences in the ratio of yolk to total contents may obscure real differences in thiamine content. However, in the fowls studied the percentages of yolk found in the eggs of the different breeds were remarkably similar.

For the White Leghorns the amount of thiamine per 100 gm. of yolk (279 µg.) was 66% greater than that in eggs of Rhode Island Reds (167 µg.) and 60% greater than the figure for Barred Rocks (175 µg.). Application of the *t* test shows (table 2) that, even with the comparatively small numbers involved, these differences are statistically significant.

TABLE 2

Tests for significance of differences between Rhode Island Reds, Barred Rocks and White Leghorn breed with respect to thiamine content of their eggs.

BREEDS COMPARED	THIAMINE PER EGG			THIAMINE PER 100 GM. EGG YOLK		
	Difference	t	p	Difference	t	p
R.I.R. — B.R.	0.9	.33	.7–.8	7.4	.72	.4–.5
W.L. — R.I.R.	14.7	6.70	$<$ .01	111.7	8.73	$<$ .01
W.L. — B.R.	15.6	7.07	$<$ .01	104.3	8.09	$<$ .01
W.L. — both heavy breeds combined	15.2	8.43	$<$ .01	107.8	11.18	$<$ .01

Whether the thiamine be measured as the amount per egg or as the amount per 100 gm. of yolk, the small differences between the Rhode Island Reds and the Barred Rocks were quite insignificant. In contrast, the values for p of $< .01$ in all the comparisons of White Leghorns with the two heavy breeds show that the excess of thiamine in the Leghorn eggs is highly significant. In the eggs of twenty-three different White Leghorn hens, the thiamine content varied from 214 to 360 µg. per 100 gm. yolk, but in eggs from twenty-two birds of the heavy breeds the range was only from 127 to 200 µg. except for a single egg with an assay of 235 µg.

It should be pointed out that the Leghorns laid somewhat smaller eggs than did the other two breeds, because they had not been laying quite as long as the others. Experience with eggs of these breeds at Cornell has demonstrated that the eggs of White Leghorns laying at maturity are not smaller despite the smaller size of the birds.

The yolk weight was also smaller in the Leghorn eggs than in either of the other two breeds by a statistically significant amount. Since all of the thiamine is contained in the yolk, this should mean less thiamine

in the eggs of White Leghorns. Similarly the heavy breeds would be expected to show more thiamine per egg because of the slightly larger yolks. When the correlation between the thiamine per egg and the weight of the yolk is examined the following coefficients are found: in Leghorns $r = +0.232$, $p = 0.2–0.3$; in Rhode Island Reds and Barred Rocks combined $r = +0.728$, $p = < 0.01$.

While the correlation in the heavy breeds is highly significant statistically, that for the Leghorns is less so. However, both indicate that the amount of thiamine increases with yolk size. The observation that the somewhat smaller yolks of the Leghorns contained more thiamine than the larger yolks of the heavy breeds is all the more striking, because it is in contrast to the relationship shown by these correlations.

TABLE 3

Thiamine content of White Leghorn eggs from a poultry farm.

DATE	EGGS IN SAMPLE NUMBER	MEAN WEIGHT OF CONTENTS IN GRAMS	THIAMINE IN MICROGRAMS	
			per egg	per 100 gm. egg
Jan. 21, 1944	24	54.8	48.2	88
Feb. 24, 1944	36	56.2	51.1	91
Apr. 1, 1944	15	54.6	59.5	109
Oct. 12, 1944	10	50.3	48.3	96
Oct. 27, 1944	10	42.6	42.6	100
Jan. 1, 1945	20	47.6	44.3	93
Jan. 29, 1945	10	51.8	37.3	72
Feb. 8, 1945	10	50.7	40.6	80
Mar. 10, 1945	10	48.5	48.5	100
Apr. 12, 1945	10	50.6	50.6	100
Averages, these Leghorns:		50.8	47	93
Averages, Cornell Leghorns:		47.0	49	105

The question arises whether the comparisons of the breeds might be influenced by the fact that the Barred Rocks and Rhode Island Reds had been laying about 6 weeks longer than the Leghorns when the eggs were taken for analysis. This possibility seems unlikely, because the amount of thiamine in the diet was too far in excess of the bird's requirements to permit any depletion of reserves in the body. Moreover, a series of determinations on White Leghorn eggs from a nearby poultry farm (table 3) showed concentrations of thiamine quite comparable with those for the Cornell Leghorns. None of them approached the low content found in eggs of Barred Rocks and Rhode Island Reds.

These analyses were made at different seasons in the course of 2 years and with the supply flocks in different stages of egg production. Since the seasonal variations are comparatively small, it seems un-

likely that the differences shown in tables 1 and 2 were influenced by anything other than the genetic constitutions of the breeds involved.

DISCUSSION

While the difference between White Leghorns and the other two breeds with respect to the thiamine content of their eggs is quite distinct, the physiological basis for this is not so clear. Ellis and workers ('33) have shown that fowls consuming diets high in vitamin B_1 put from two to three times as much of it in their eggs as do fowls on diets low in that vitamin. Their data suggest that the amount of thiamine found in the egg is some indication of the excess present in the body above the level required for normal physiological processes. If this be so, then among fowls getting the same diet, the ones utilizing the most thiamine for their metabolic processes (in this case, the two heavy breeds) would have the least surplus available for deposition in the egg. Conversely, the comparatively high content of thiamine in eggs of the White Leghorns should indicate a lower requirement for the vitamin in the normal metabolism of this breed.

If the White Leghorns consumed more feed the difference would not be as striking. On the contrary the differences shown in tables 1 and 2 are emphasized by the fact that the total thiamine intake, which is directly related to the amount of feed consumed, was less for the White Leghorns than for the two heavy breeds. The latter were found in extensive comparisons by Waite ('34) to consume about 13 to 15% more feed per year than White Leghorns. These differences would undoubtedly have been still greater if the Reds and Rocks had laid another thirteen to twenty eggs per bird to equal the egg production of the Leghorns. The contrast is also emphasized by the fact that the White Leghorns are known to be more active than hens of the heavy breeds. On this basis they should use more thiamine and have less to deposit in their eggs.

The difference is not due to the greater body mass of the heavy breeds. The average weight of the Leghorns in the flock laying the eggs studied was 1941 gm. in January, 1945. At the same time, the weight of the Rhode Island Reds would be about 2700 gm. and of the Barred Rocks about 2540 gm.[4] The 23–24% greater body mass of the heavy breeds does not account for observed thiamine differences in the egg of 60–66%, particularly when the other factors discussed above are taken into account.

[4] The last two figures are not actual weights but are the averages of fifty or more representatives of each breed weighed several years ago.

Scrimshaw and co-workers ('44) have shown that the thiamine initially deposited in the egg is still present at the time of hatching. The demonstration that White Leghorn eggs contain more thiamine than those of Rhode Island Reds and of Barred Rocks suggests that the greater resistance of Leghorn chicks to a deficiency of thiamine found by Lamoreux and Hutt ('39) might have resulted solely from the greater concentration in their tissue at hatching. This error could not apply to the trials in which all chicks received a normal diet, high in thiamine, before being put on the deficient diets at 2 and 3 weeks of age. In those chicks, as well as in the adult fowls studied by the Roumanian workers, the White Leghorns were consistently more resistant to a deficiency of vitamin B_1 than the other breeds. From these earlier comparisons on deficient diets, together with the present one on diets with ample thiamine, it is clear that White Leghorns must differ from Rhode Island Reds and from Barred Rocks in being able to utilize more efficiently the thiamine in their diets.

While differences between species in nutritional requirements are well known, genetic variability in this respect has been comparatively little studied within species. That strains of the rat differ in requirement of vitamin B_1 was shown by Light and Cracas ('38). In the ascomycete, Neurospora sitophila, most strains can synthesize thiamine, but Beadle and Tatum ('41) found a mutant able to synthesize only the pyrimidine half of the molecule and not the thiazole half. It seems probable that further study will bring to light other cases of genetic variability in the utilization of this vitamin.

In other comparisons with Rhode Island Reds and Barred Rocks, the White Leghorns have proven more resistant to extreme heat (Hutt, '38), more resistant to Salmonella pullorum, (Hutt and Scholes, '41) and superior in regulation of body temperature shortly after hatching (Lamoreux and Hutt, '39). In a discussion of these physiological traits and others (Hutt, '41), it was pointed out that they are inherited characteristics just as much as the more obvious variations in structure and color by which breeds are differentiated. To account for them is difficult, since all are characters for which there could have been no conscious selection by the breeders when the breeds were formed. Since the Leghorns originated in the Mediterranean area, while the ancestors of the Rhode Island Reds and Barred Rocks came from China, the possibility exists that natural selection in different environments was instrumental in bringing about the differences observed.

SUMMARY

Using a modified macro-fermentation assay procedure, the mean thiamine content of the egg was compared in three different breeds of domestic fowl. These were kept under comparable conditions and their diet contained about 5 μg. of thiamine per gram. The thiamine content was found to average 279 (in μg. per 100 gm. of yolk) for White Leghorn eggs, 167 for Rhode Island Reds and 175 for Barred Rocks (105, 64, 66 μg. per 100 gm. of egg contents, respectively).

From these results and other reports for hens on diets deficient in thiamine, it is concluded that the White Leghorns must differ genetically from the other two breeds in the capacity to utilize more efficiently the thiamine in their diet.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1938 Studies on the vitamin B_1 requirements of growing chicks. J. Nutrition, vol. 15, p. 403.

BAKER, A. Z., AND M. D. WRIGHT 1935 The vitamin B_1 content of foods. Biochem. J., vol. 29, p. 1802.

BEADLE, G. W., AND E. L. TATUM 1941 Genetic control of biochemical reactions in Neurospora. Proc. Nat. Acad. Sci., vol. 27, p. 499.

BETHKE, R. M., P. R. RECORD AND F. W. WILDER 1936 The effect of the ration of the hen on the vitamin G content of egg with observations on the distribution of vitamin B and G in normal eggs. J. Nutrition, vol. 12, p. 309.

CHICK, H., AND M. H. ROSCOE 1929 A method for the assay of the antineuritic vitamin B_1 in which the growth of young rats is used as a criterion. Biochem. J., vol. 23, p. 498.

ELLIS, N. R., D. MILLER, H. W. TITUS AND T. H. BYERLY 1933 Effect of diet on egg composition. III. The relation of diet to the vitamin B and the vitamin G content of eggs, together with observations on the vitamin A content. J. Nutrition, vol. 6, p. 243.

HUTT, F. B. 1938 Genetics of the fowl. VII. Breed differences in susceptibility to extreme heat. Poultry Sci., vol. 17, p. 454.

——— 1941 The association of physiological traits with breed characteristics in the fowl. Proc. Seventh Internat. Genetical Congr., Edinburgh, 1939. Supplement to J. Genet., p. 156.

HUTT, F. B., AND J. C. SCHOLES 1941 Genetics of the fowl. XIII. Breed differences in susceptibility to Salmonella pullorum. Poultry Sci., vol. 20, p. 342.

LIGHT, R. F., AND L. J. CRACAS 1938 Vitamin B requirements of different strains of white rats. Science, vol. 87, p. 90.

LAMOREUX, W. F., AND F. B. HUTT 1939 Breed differences in resistance to a deficiency of vitamin B_1 in the fowl. J. Agric. Res., vol. 58, p. 307.

——— 1939 Variability of body temperature in the normal chick. Poultry Sci., vol. 18, p. 70.

NICHITA, G., AND G. IFTIMESCO 1934 Recherches sur l'avitaminose du complex B chez les poules Leghorn blanches. Ann. Inst. Natl. Zootech. Roumanie, vol. 3, p. 79. (Also in Compt. Rend. Soc. Biol. (Paris), vol. 117, p. 289, 1934.)

NICHITA, G., N. TUSCHAK AND C. CALCEF 1934 Recherches sur l'avitaminose du complex B chez les poules Rhode Island Red. Ann Inst. Natl. Zootech. Roumanie, vol. 3, p. 35. (Also in Compt. Rend. Soc. Biol. (Paris), vol. 117, p. 283, 1934.)

SCHULTZ, A. S., L. ATKIN AND C. N. FREY 1942 Determination of vitamin B_1 by yeast fermentation method. Improvements related to use of sulfite cleavage and a new fermentometer. Ind. and Eng. Chem., Anal. Ed., vol. 14, p. 35.

SCRIMSHAW, N. S., AND W. B. STEWART 1944 Use of the macrofermentation method for thiamine assay. J. Biol. Chem., vol. 155, p. 79.

SCRIMSHAW, N. S., W. P. THOMAS, J. W. MCKIBBEN, C. R. SULLIVAN AND K. C. WELLS 1944 Thiamine content of hen eggs during incubation. J. Nutrition, vol. 28, p. 235.

WAITE, R. H. 1934 Feed consumption studies based on six Maryland egg laying contests. Maryland Agr. Exp. Sta. Bull. no. 359, p. 291.

PHYSIOLOGICAL AVAILABILITY TO THE RAT OF CRUDE CAROTENE IN VEGETABLES

BERNARD L. OSER AND DANIEL MELNICK
Food Research Laboratories, Inc., Long Island City

(Received for publication June 9, 1945)

Using the dark adaptation test as a measure of carotene utilization by man Booher and associates ('39 a, b) found that the carotene in spinach was approximately 1.7 times as effective as that contained in peas while the carotene in both of these vegetables was utilized better than when taken in cottonseed oil. Carotene was less efficiently absorbed from carrots than from butter in the human intestinal tract (Kreula and Virtanen, '39).

Lease and co-workers ('39) and Sherman ('40, '41) have observed that the utilization of carotene by the rat in curative growth tests is a function of the solvent. Smith and Otis ('41) reported that the quantity of vitamin A in the livers of depleted rats fed carotene varied with the source. Using essentially the same technic Guggenheim ('44) has demonstrated that the utilization of carotene derived from various plant materials ranged between 33 and 67% of that noted when preformed vitamin A was taken. In the case of lettuce, however, carotene utilization was found to equal that of the preformed vitamin.

Studies during the past several years (Bacharach, '40; Davies and Moore, '41; and Harris and associates, '43) have demonstrated that vitamin E (tocopherol) improves utilization of carotene by protecting it against oxidation primarily in the intestinal tract. In this connection it is of interest that of the vegetables tested lettuce was found to contain the highest amount of vitamin E (Karrer and Keller, '38). Guggenheim ('44) was able to demonstrate that supplementation of the basal carrot diet with tocopherol doubled the biological value of the carotene content. These findings suggest that the variability in responses following the ingestion of carotene derived from different food sources, reported for both the rat and the man, may be due in part to variations in tocopherol intake.

In the present study results are reported on a variety of vegetable products correlating the colorimetrically determined crude carotene

content with biological vitamin A potency. Tocopherol was ruled out as a variable in the biological assays.

EXPERIMENTAL PART

Assay materials. These were canned vegetables prepared from one batch and stored during the period of assay at 5°C. For an evaluation of the physiological availability of carotenes derived from a variety of vegetables it was considered desirable to use canned pureed or chopped vegetables rather than raw products. This avoided uncertainty regarding either the uniformity of the assay doses or the stability of the carotenoids, inasmuch as no active enzymes remained in the heat-processed foods.[1]

Assay procedures. A photometric adaptation of the A.O.A.C. ('40) colorimetric procedure was employed for estimating crude carotene. The standard was β-carotene, recrystallized according to the method of Kemmerer ('41). The test is conducted on the unsaponifiable fraction after the removal of the xanthophyls by extraction of the petroleum ether solution with 90% methanol. When tomatoes were present, the lycopene and xanthophyl in the unsaponifiable extract were first removed by adsorption on specially prepared magnesium carbonate (Fraps and associates, '40). In the International Standard for vitamin A, one unit is 0.6 μg. of pure β-carotene. However, in setting the feeding levels for the bioassays 1 μg. of crude carotene was assumed to have the potency of one unit of vitamin A. Once each week a fresh can was opened for preparation of the assay supplements. The remaining portion was stored at 5°C. under nitrogen. Tocopherol was eliminated as a variable in the bioassays by the daily administration to each rat of 0.1 ml. of cottonseed oil containing 0.3% mixed tocopherols.[2] Since approximately 0.2 mg. tocopherol was supplied daily via the cottonseed oil in the basal diet, the rats received a total of approximately 0.5 mg. tocopherol per day. The results of recent studies (Harris and associates, '43) indicate that the optimal effect of tocopherol supplementation of the basal ration is attained at the level of 0.5 mg. per day.

In order to permit estimates of potency to be calculated from the animal data, the USP reference cod liver oil was fed at three levels, 2, 3 and 4 units per day, and the assay material at an assumed level cor-

[1] Colorimetric carotene analyses (A.O.A.C., '40) of the material removed from the commercial container before and after storage in jars for periods of 2 weeks at 5°C. gave the same values, indicating no loss of the provitamin.

[2] Obtained from Distillation Products, Inc., Rochester, New York. In the fortification of the cottonseed oil correction was made for the purity of the mixed tocopherol solution.

responding to the median dose, 3 units. Each estimate of potency was based on interpolation of the assay response on the curve for the comparable reference groups.

RESULTS

Eighteen samples of ten vegetable products were tested by the colorimetric and biological assay procedures. The results listed in table 1 show good parallelism between the bioassay data and the vitamin A values predicted by the non-biological method. On the average, 1.0 µg. of crude carotene (free from xanthophyl and lycopene) in these vegetables was found to be equivalent to one USP unit of vitamin A.

TABLE 1

Comparable vitamin A activity of carotenes in a variety of heat-processed vegetables.

MATERIAL	SAMPLE NO.	CAROTENE CONTENT [1] (A)	BIOASSAY [2] ESTIMATE OF VITAMIN A POTENCY (B)	RATIO (A/B)
		µg./gm.	*USP units/gm.*	
Carrots	1	62	64	1.0
	2	32	36	0.9
Kâle — swiss chard	3	17	17	1.0
	4	20	19	1.1
Mixed greens [3]	5	17	18	0.9
	6	14	18	0.8
Prunes	7	5	6	0.8
Pumpkin	8	25	28	0.9
Spinach	9	29	27	1.1
	10	34	36	0.9
Squash	11	25	17	1.5
Squash — pumpkin	12	19	17	1.1
Tomato [4] product	13	15	14	1.1
Vegetable soups	14	25	32	0.8
	15	20	23	0.9
	16	18	23	0.8
	17	14	16	0.9
	18	13	17	0.8
Mean ratio of carotene content to vitamin A potency				1.0

[1] Crude carotene, free from xanthophyls, estimated colorimetrically (A.O.A.C., '40).

[2] Based on the procedure of the U. S. Pharmacopoeia XII ('42).

[3] Kale, swiss chard and lettuce.

[4] The lycopene and xanthophyl in the unsaponifiable extract were removed by adsorption on a specially prepared magnesium carbonate (Fraps, Kemmerer and Greenberg, '40).

In the case of two fruit products, an apricot-apple mixture and a peach purée, the biological responses were found to be far in excess of those predicated upon the carotene values. The bioassay estimate for the former product was 25 units per gram while the latter was found to contain 12 units per gram. The colorimetric analyses indicated that there were only 4 μg. carotene per gram in each of these products. Since the responses by the animals to the materials fed at the levels suggested by the colorimetric tests were far in excess of that obtained with the maximal dose of the USP reference oil, the assays were repeated at higher assumed levels of vitamin A content. The responses then fell within the reference points obtained with the USP oil so that these bioassay estimates are considered to be trustworthy. The colorimetric values were confirmed by replicate analyses. The results obtained with these two materials indicate that the crude carotene content of fruit products may not be a reliable index of vitamin A potency. Other substances may be present which have vitamin A activity but which are either not initially extracted or are removed by the aqueous or 90% methanol washings of the unsaponifiable extract, or do not absorb light at 450 millimicrons. This problem merits further study.

SUMMARY

In testing heat-processed vegetables good correlation is obtained between the bioassay estimates of vitamin A potency and those predicted from the results of colorimetric analyses for crude carotene, provided that in the rat assay the tocopherol variable is eliminated. In these products 1.0 μg. of crude carotene (free from xanthophyl and lycopene) is equivalent to one USP unit of vitamin A. No great differences are observed in the physiological availability of the carotene derived from these vegetable products. In the case of some fruit products the potency predicted from the crude carotene values may significantly underestimate the biological vitamin A activity.

LITERATURE CITED

BACHARACH, A. L. 1940 Effect of ingested vitamin E (tocopherol) on vitamin A storage in the liver of the albino rat. Quart. J. Pharm., vol. 13, p. 138.

BOOHER, L. E., AND E. C. CALLISON 1939 a The minimum vitamin A requirement of normal adults. II. The utilization of carotene as affected by certain dietary factors and variations in light exposure. J. Nutrition, vol. 18, p. 459.

BOOHER, L. E., E. C. CALLISON AND E. M. HEWSTON 1939 b An experimental determination of the minimum vitamin A requirements of normal adults. J. Nutrition, vol. 17, p. 317.

DAVIES, A. W., AND T. MOORE 1941 Interaction of vitamins A and E. Nature, vol. 147, p. 794.

FRAPS, G. S., A. R. KEMMERER AND S. M. GREENBERG 1940 Determination of carotene in presence of lycopene. J. Assn. Off. Agri. Chem., vol. 23, p. 422.

GUGGENHEIM, K. 1944 The biological value of carotene from various sources and the effect of vitamin E on the utilization of carotene and of vitamin A. Biochem. J., vol. 38, p. 260.

HARRIS, P. L., M. W. KALEY AND K. C. D. HICKMAN 1943 Covitamin studies. II. The sparing action of natural tocopherol concentrates on carotene. J. Biol. Chem., vol. 152, p. 313.

KARRER, P., AND H. KELLER 1938 Quantitative Bestimmung der Tocopherole in verschiedenen Ausgangsmaterialien. Helv. Chim. Acta, Bd. 21, S. 1161.

KEMMERER, A. R. 1941 Report on carotene. J. Assn. Off. Agri. Chem., vol. 24, p. 859.

KREULA, M., AND A. I. VIRTANEN 1939 Absorption of carotene from carrots in human subjects. Upsala LäKarefören. Förh., vol. 45, p. 355.

LEASE, E. J., J. G. LEASE, H. STEENBOCK AND C. A. BAUMANN 1939 The biological value of carotene in various fats. J. Nutrition, vol. 17, p. 91.

Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists 1940.

SHERMAN, W. C. 1940 Chromatographic identification and biological evaluation of carotene from mature soybeans. Food Res., vol. 5, p. 13.

———— 1941 The effect of certain fats and unsaturated fatty acids upon the utilization of carotene. J. Nutrition, vol. 22, p. 153.

SMITH, M. C., AND L. OTIS 1941 Observations on carotene analysis of vegetables and fruits as a basis for prediction of their vitamin A value. Food Res., vol. 6, p. 143.

U. S. PHARMACOPOEIA 1942 Twelfth Edition.

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1946 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1946.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1945 must be in the hands of the Secretary by January 10, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

H. E. CARTER
Noyes Laboratory of Chemistry
University of Illinois
Urbana, Illinois

SECRETARY, AMERICAN INSTITUTE OF NUTRITION

BORDEN AWARD IN NUTRITION

The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1946. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

W. E. KRAUSS
Ohio Agricultural Experiment Station
Wooster, Ohio

CHAIRMAN, NOMINATING COMMITTEE

NICOTINIC ACID AND THE LEVEL OF PROTEIN INTAKE IN THE NUTRITION OF THE PIG[1]

MAXWELL M. WINTROBE,[2] HAROLD J. STEIN, R. H. FOLLIS, JR. AND STEWART HUMPHREYS[2]

Departments of Medicine, Johns Hopkins University, Baltimore, and University of Utah, Salt Lake City

THREE FIGURES

(Received for publication July 9, 1945)

Shortly following discovery of the action of nicotinic acid in "black tongue" in dogs (Elvehjem et al., '38), Chick and co-workers ('38) reported the dramatic effect brought about by the administration of nicotinic acid to pigs which had been fed a diet consisting of white maize, peameal, purified casein, cod liver oil, and salt mixture. A need for nicotinic acid on the part of the pig was also observed by Hughes ('38), Madison, Miller and Keith ('39), and in our laboratory (Wintrobe, '39).

In all of the experiments cited above, the diets used were no doubt incomplete in several respects. Although the use of nicotinic acid produced a marked improvement in the growth and appearance of pigs receiving only thiamine and riboflavin, it was soon evident in our own studies that additional vitamins are required by these animals. Recently we showed (Wintrobe, Miller, Follis et al., '42) that excellent growth and development take place when supplements of pyridoxine, choline, and calcium pantothenate are furnished in addition to thiamine, riboflavin, and nicotinic acid. It was then that an attempt was made to find out what the effect in pigs is when, of the above six vitamins only nicotinic acid is omitted. To our surprise, no ill effects were observed.

The basal diet used by Chick et al. ('38) was relatively low in protein (about 14%) and probably lacked certain B vitamins other than nicotinic acid (Waisman and Elvehjem, '40). This was true also of the diet used by Hughes ('38). Similar deficiencies may have been present in

[1] These studies were aided by grants from the Rockefeller Foundation, the Fluid Research Fund of the Johns Hopkins Medical School, Parke, Davis and Company and the Upjohn Company, and were carried out in cooperation with the Bureau of Animal Industry, U. S. Department of Agriculture.

[2] Present address, School of Medicine, University of Utah, Salt Lake City, Utah.

the diet of Madison et al. ('39), although exact information is lacking. Our animals (Wintrobe, '39) were fed as much as 26.1% casein, but the diet was supplemented only with thiamine and riboflavin and was deficient at least in pyridoxine (Wintrobe, Follis, Miller et al., '43) and pantothenic acid (Wintrobe, Follis, Alcayaga et al., '43) as well as in nicotinic acid. It is of interest in this connection to note that the diet of the pellagrin is poor in protein and minerals as well as in vitamins and that the Goldberger experimental diet supplies protein which is poor qualitatively as well as quantitatively.

In view of these facts it seemed possible that, when of the known dietary factors required by the pig nicotinic acid alone was not furnished, our failure to note any ill effects might be explained by some alteration in the requirement for nicotinic acid under the conditions of our experiment. In particular it seemed possible that the high level of protein in the diet might be a factor of importance.

EXPERIMENTAL PROCEDURE

The animals described in this study consisted of two series. The more important one consists of seventeen pigs derived from five litters, all of which were received at the same time and were placed on experiment at approximately the same age (69 to 77 days of age). These pigs were divided into six experimental groups as evenly as possible, having in mind litter and weight. The remainder, which comprise forty-four pigs, are additional animals studied from time to time which furnish supplementary data concerning the role of nicotinic acid in the nutrition of the pig. These also include litter mates divided among various experimental groups. The total of sixty-one pigs make up nine groups of pigs and several subgroups, as shown in table 1.

The "high protein" diet is our standard basal diet, already described (Wintrobe, Miller, Follis et al., '42) of which 26.1% is "crude" casein, the remainder consisting of sucrose, lard, and a salt mixture. In the "low protein, high fat" diet, the lard was increased to make up for the reduction of casein, as follows: crude casein, 10.0%; sucrose, 56.8%; lard, 26.8%; and salt mixture, 6.4%. In the "low protein, low fat" diet, sucrose was increased to make up for the reduction of casein, as follows: crude casein, 10.0%; sucrose, 73.8%; lard, 11.0%; and salt mixture, 5.2%. The diets were fed in such quantities that the caloric intake for all the animals was the same.

The Goldberger diet consisted of ground white maize (76.4%), peameal (10.3%), crude casein (6.4%), cod liver oil (4.4%), and salt mix-

ture (2.5%). The protein content of this mixture was approximately 17.3%.

All diets were supplemented with cod liver oil or blended fish oils,[3] 0.5 gm. per kilogram body weight daily, as in experiments previously described (Wintrobe, Miller, Follis et al., '42).

TABLE 1

Average daily weight gain of pigs fed various experimental diets.

GROUP NO.	BASAL DIET				CRYSTALLINE VITAMIN SUPPLEMENTS [1]	NO. OF PIGS	AGE EXPERIMENT		AVERAGE DAILY WEIGHT GAIN
	Casein	Fat	Carbo-hydrate	Type of casein			Begun on days – to –	Ended on days – to –	
	%	%	%						gm.
1	26.1	11.0	57.7	"Crude"	T	5	67–123	119–247	107
2	26.1	11.0	57.7	"Crude"	TR	4	67– 80	106–237	108
						2	16– 22	78–122	67
						2	34	99–128	42
3	26.1	11.0	57.7	"Crude"	TRN	11	64–107	87–300	123
						2	16– 22	72– 74	58
4	26.1	11.0	57.7	"Crude"	TR ChP	3	70– 77	118–238	154
5	26.1	11.0	57.7	"Crude"	TR B_6ChP	6	70– 90	210–292	413
5a	26.1	11.0	57.7	Vitamin-free [2]	TR B_6ChPIp	2	16– 22	136–225	360
6	26.1	11.0	57.7	"Crude"	TRNB_6ChP	9	70–106	216–448	336
6a	26.1	11.0	57.7	"Crude"	TRNB_6ChPIp	1	34	213	433
6b	26.1	11.0	57.7	Vitamin-free	TRNB_6ChP	3	87– 88	236–238	281
7	10.0	26.8	56.8	"Crude"	TR B_6ChP	3	69– 77	134–227	51
7a	10.0	11.0	73.8	"Crude"	TR B_6ChPIp	2	21– 22	65–134	51
8	10.0	26.8	56.8	"Crude"	TRNB_6ChP	3	69– 77	95–225	162
9	17.3	7.4	75.3	"Crude" (Goldberger)[3]	TR B_6ChP	3	71– 77	114–230	167

[1] T refers to thiamine, 0.25 to 0.52 mg. per kg. body weight daily; R, riboflavin, 0.12 mg.; N, nicotinic acid, 1.20 mg.; B_6, pyridoxine hydrochloride, 0.20 mg.; Ch, choline chloride, 10.0 mg.; P, calcium pantothenate, 0.50 mg.; I, inositol, 1.20 mg.; p, para-aminobenzoic acid, 0.50 mg.

[2] From SMA Corporation, Cleveland, Ohio.

[3] "Goldberger" diet: ground white maize, 29.8 gm.; peameal, 4.0; crude casein, 2.5; cod liver oil, 1.7; salt mixture, 1.0 gm. Protein consists of casein, 6.5%; vegetable, 10.8%.

Complete details concerning the management of the animals have been published elsewhere (Wintrobe, Miller, Follis et al., '42; Wintrobe, Miller and Lisco, '40). The data presented in this report have been gathered since 1937 (Wintrobe, Mitchell and Kolb, '38). Throughout this period, the source of the animals, their handling, and their diet, even including the source of the casein fed, were not changed. The only change that was made was in the mineral mixture. The one first used (Wintrobe, Mitchell and Kolb, '38) was modified in June, 1939, to in-

[3] Mead Johnson and Company, Evansville, Ind.

clude copper, manganese, zinc, and cobalt, and ferric pyrophosphate replaced ferrous sulphate (Wintrobe, Miller and Lisco, '40).

The pigs were kept in specially constructed metabolism cages designed to minimize contact of urine with feces.

The procedure in these studies was to feed pigs the basal ("high protein") diet when they were received (about 21 days of age) together with 3 gm. of yeast per kilogram body weight daily. When the experimental period was commenced, the only source of the B complex, aside from the minimal amounts possibly present in the basal diet, was in the form of the crystalline vitamin supplements specified for each group.

In comparing the various animals, attention should be paid to two important factors, namely, litter mates and the time when an experiment was begun. The latter factor is important since older animals had a better opportunity to store a supply of all the nutritive factors present in yeast than those started on a mixture of crystalline vitamins at a very early age.

From this viewpoint the observations on the following seventeen pigs, referred to above, are to be considered particularly significant: Litter XXX, pigs 6-87, 7-02 and 6-81 (groups 4, 8 and 9); litter XXXI, pigs 6-84, 6-93 and 6-99 (groups 5, 6 and 7); litter XXXII, pigs 6-88, 6-85, 7-00, 7-04, 7-06, 6-82 and 6-83 (groups 4, 5, 7, 8 and 9); litter XXXIV, pigs 6-89 and 6-95 (groups 4 and 6); litter XXXV, pigs 6-86 and 7-01 (groups 5 and 7). Also worthy of special attention are the pigs of the following litters which are to be found in a variety of experimental groups: Litter XXVI, pigs 6-55, 6-51, 6-59, 6-57 (groups 3, 5, 6, 6b); litter XXVII, pigs 6-68, 6-63, 6-65, 6-61 (groups 3, 5, 6, 6b); litter XXVIII, pigs 6-69, 6-72, 6-75, 6-78 (groups 3, 5, 6, 6b); litter XLIII, pigs 7-67, 7-69, 7-73, 7-71 (groups 2, 3, 5a, 7a); litter XLIX, pigs 7-68, 7-70, 7-74 (groups 2, 3, 5a).

RESULTS

The role of nicotinic acid. The data for growth are summarized in table 1 and the growth curves for the individual pigs are shown in figure 1. Comparison of groups 1 and 2 with the eleven pigs of group 3 which received crystalline vitamin supplements and no yeast at a correspondingly late age (64 to 107 days), reveals a somewhat better growth rate for the animals receiving nicotinic acid in addition to thiamine and riboflavin, as compared with those receiving only the last two vitamins. The animals receiving nicotinic acid not only were larger than the pigs not receiving this vitamin (fig. 2) but their coats were in better condition, their muscles were less flabby, and there was less diarrhea. These

pigs, however, were not litter mates. Two pigs (7-69, 7-70) were fed no yeast when they were received, but were given thiamine, riboflavin, and nicotinic acid at once (16 to 22 days of age). These animals grew very poorly, and no better than their litter mates who were given only thiamine and riboflavin at the same age (7-67, 7-68).

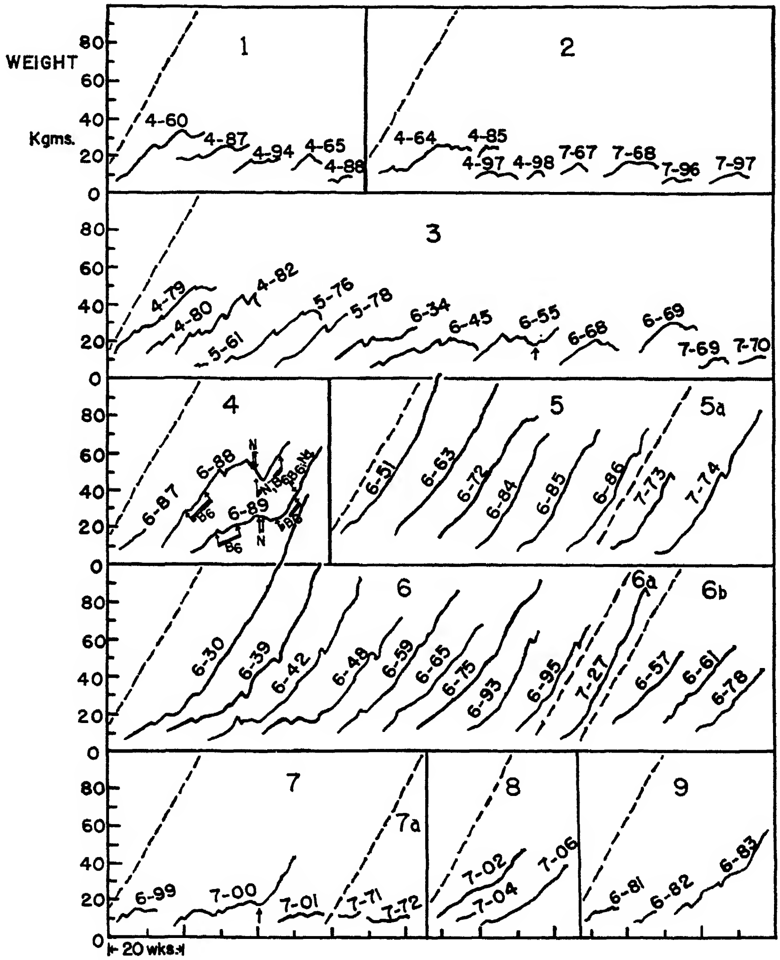


Fig. 1 Growth curves of pigs in various experimental groups. Note that the growth of pigs fed a high protein diet and receiving no nicotinic acid (group 5) was as good as that of pigs fed this vitamin (group 6) even when pigs given vitamin-free casein are compared (groups 5a and 6b). However, when the basal diet was low in protein, the growth of pigs given no nicotinic acid (groups 7 and 7a) was not as good as that of pigs receiving this vitamin (group 8).

Arrows indicate restoration of normal (high protein) diet and feeding of all necessary vitamin supplements to pigs 6-55 (group 3) and 7-00 (group 7). Pigs 6-88 and 6-89 (group 4) were given, from time to time, pyridoxine (B_6) and nicotinic acid (N).

The pigs mentioned above as developing relatively well when the experimental deficiency was commenced at 2 or 3 months of age instead of at 3 weeks, failed eventually also. Their growth became impaired, diarrhea persisted, anemia, epileptiform convulsions, and marked ataxia together with extensive degenerative changes in the nervous system developed. We have been able to show that these symptoms resulted from deficiencies of pyridoxine (Wintrobe, Follis, Miller et al., '43) and of pantothenic acid (Wintrobe, Follis, Alcayaga et al., '43).

When pyridoxine, calcium pantothenate, and choline were furnished in addition to thiamine, riboflavin, and nicotinic acid, animals receiving the same basal diet as those in groups 1, 2 and 3 grew very well, and no pathological changes occurred (group 6). The addition of inositol and p-aminobenzoic acid to the group of six vitamins mentioned above was associated with better growth in one animal (group 6a) as compared with group 6 as a whole. This difference, however, may not be significant for three of the nine pigs in group 6 (6-30, 6-93, 6-95) grew almost as well as this pig (average daily weight gain, 400, 396 and 394 gm., respectively). Good, though somewhat less satisfactory growth occurred in three pigs fed vitamin-free (group 6b) instead of crude casein (group 6) in association with the six "B" vitamins (compare litter mates 6-59 and 6-57, 6-65 and 6-61, 6-75 and 6-78).

Comparable animals given the same basal diet and the same vitamin supplements as those mentioned above (group 6) except that no nicotinic acid was supplied (group 5) showed no signs of nutritional deficiency except for slightly less satisfactory growth in certain instances as compared with those given this vitamin, and at autopsy no pathological changes attributable to deficiency were found. In table 2 data for comparable animals are presented and weight gain, expressed in terms of percentage of initial weight, is shown.

Neurological changes were absent. In none of the pigs of groups 5, 5a, 6, 6a or 6b did anemia or other morphological changes in the blood develop. When pyridoxine also was omitted (group 4), the changes which occurred (impaired growth, microcytic anemia, epileptiform convulsions, sensory neuron degeneration) were the same as those which develop in pyridoxine deficiency alone (Wintrobe, Follis, Miller et al., '43).

It is difficult to determine from our data whether "crude" casein supplied an unknown factor making it possible for pigs to dispense with dietary nicotinic acid. The pigs of group 5a grew well, even though they received vitamin-free casein. Nevertheless a direct comparison with the pigs of group 5 is not possible since groups 5 and 5a did not

contain litter mates. Furthermore, the two pigs of group 5a were given inositol and p-aminobenzoic acid in addition to the five vitamins furnished group 5, and this may or may not have contributed to the excellent growth of the pigs in group 5a.

From the standpoint of storage of nicotinic acid as a factor in influencing the need for dietary nicotinic acid, it is to be noted that the animals in group 5a received the crystalline vitamin supplements at a very early age (16 to 22 days) without opportunity to store growth factors available in yeast. In this respect they were at a disadvantage as compared with the pigs of group 5, for storage might have occurred in the case of the latter pigs. In any event, the excellent growth of the pigs

TABLE 2

Comparison of growth in individual animals.

GROUP 5 "High protein, no nicotinic acid"			GROUP 6 "High protein with nicotinic acid"		
Pig no.	Initial weight	Weight gain	Pig no.	Initial weight	Weight gain
	kg.	*%*		*kg.*	*%*
6-51	16.0	657	6-59	9.0	978
6-63	14.5	675	6-65	10.1	673
6-72	13.2	606	6-75	11.1	840
6-84	11.7	570	6-93	10.9	608
6-85	11.6	640	6-95	10.4	648
6-86	11.0	654			
GROUP 7 "Low protein, high fat, no nicotinic acid"			GROUP 8 "Low protein, high fat, with nicotinic acid"		
6-99	11.6	128	7-02	15.8	296
7-00	10.4	179	7-06	8.6	459
7-01	10.5	127	7-04 [1]	10.2	116

[1] Died after only 26 days on experiment.

given vitamin-free casein but no nicotinic acid (group 5a) makes it clear that under the experimental conditions described, the pig can grow well in the absence of nicotinic acid.

The role of protein. When animals receiving the "low protein" diet were given all of the vitamins already mentioned with the exception of nicotinic acid (groups 7 and 7a), growth was extremely poor (fig. 3). The coats of the pigs were rough and untidy. Diarrhea and poor appetite developed in all three pigs of group 7 ("low protein, high fat, no nicotinic acid"), and severe normocytic anemia also occurred, the red cell counts being 1.88, 2.33, and 3.54 millions, respectively, per cubic millimeter. Furthermore, although no definite abnormality in gait was observed during life, slight degenerative changes were found in the

posterior root ganglion cells of two of the three animals in group 7 and rather extensive chromatolysis in the dorsal root ganglia was present in the third. Only one of the pigs of group 7a ("low protein, low fat, no nicotinic acid") developed severe diarrhea. There was little anemia in either of these pigs. In the nervous systems of these two pigs, rather

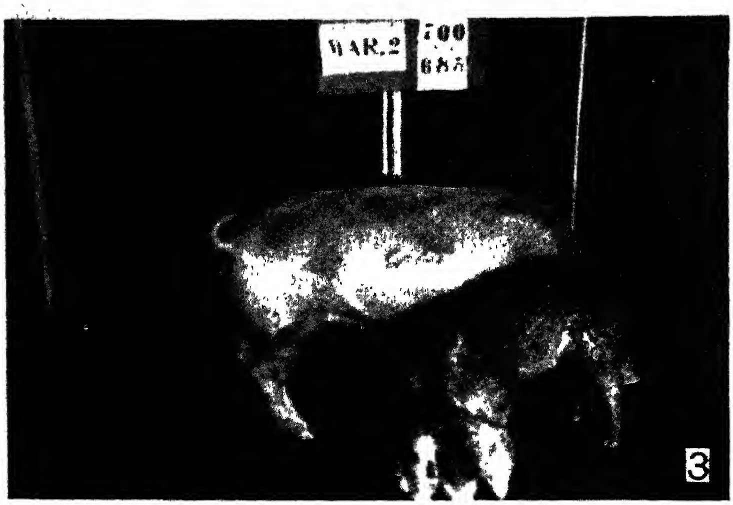


Fig. 2 Comparison of a pig of group 2 (4-98) with one of group 3 (4-82). The larger of the two pigs received nicotinic acid in addition to thiamine and riboflavin as supplements to the high protein basal diet. The smaller pig was given no nicotinic acid.

Fig. 3 Comparison of a pig of group 5 (6-85) with one of group 7 (7-00). Neither of these pigs was given nicotinic acid, but the smaller one was fed a diet low in protein. These pigs were litter mates.

extensive chromatolysis was found in the dorsal root ganglion cells of one (7-71) but there was no definite myelin degeneration. In the second pig, 7-72, the nervous tissues showed no lesions. It is interesting to note that the degenerative changes when present were found in the small ganglion cells. At autopsy extensive infections were found in the pigs of group 7, but not in group 7a. The cause of death in the two animals of the latter group was not clear.

Pigs fed a low protein, high fat diet, but furnished nicotinic acid (group 8) looked much better nourished than the pigs described above, even though their growth was impaired as compared with that of pigs given a greater proportion of protein (group 6). The growth curves of litter mates 7-00 and 7-06 of groups 7 and 8 may be compared with one another. Pigs 6-99, 7-02 and 7-01 came from other litters but were studied at the same time and under the same conditions as pigs 7-00 and 7-06. The coats of the animals in group 8 were clean, their hair was in good condition, no diarrhea developed, and only very slight normocytic anemia occurred (R.B.C. 5.90, 6.50 and 5.40, respectively, as compared with values of 6.50 million or higher in the pigs of groups 6). No pathological changes in the nervous system or other tissues were found at autopsy. Weight gain comparisons for the individual animals are presented in table 2.

One pig in group 7 (pig 7-00) was given 100 mg. nicotinic acid daily. The diarrhea ceased, the appearance of the pig improved, and its weight increased. The anemia was not altered. When the low protein diet was changed to the high protein type, further weight gain occurred, but the anemia persisted. It was not possible to influence the anemia appreciably even by the administration of liver extract or desiccated whole liver. When this animal was killed, severe infection was found (multiple abscesses: subcutaneous, lung, liver, spleen, peritoneum). It is possible that infection may have been at least in part responsible for the anemia noted in group 7, and it is likely that infection interfered with a hematopoietic response which might have occurred in pig 7-00. Therapy was attempted only in one other instance, pig 7-01 of group 7. Casein, 10 gm. per kilogram body weight daily, was fed in addition to the regular diet. No benefit was observed, but here again infection (lobular pneumonia) was found when the pig died 23 days later.

Two of the three pigs given the Goldberger ration supplemented with B vitamins except nicotinic acid (group 9) died early with widespread infection. Both had severe normocytic anemia (R.B.C. 2.69, 3.80 million, respectively). Their growth had been poor (103 and 130 gm. average daily weight gain, respectively), but their gait was normal. Extensive

degenerative changes were found in the posterior root ganglion cells, but the changes were confined to the small cells. One pig (6-83) survived a long time, grew moderately well, and had only slight anemia (R.B.C. 5.70 million). The better growth of this animal (230 gm. average daily weight gain) as compared with that of a litter mate (7-00) given no nicotinic acid and a low protein diet (group 7) may have been due to the higher content of protein in the Goldberger diet (6.5% casein, 10.8% vegetable protein, as compared with 10% casein). Pathological changes were found in the spinal ganglia in this animal, as in its mates, but the changes were not extensive. Lobular pneumonia was also present at autopsy.

Chemical studies. In an attempt to assess the degree of nicotinic acid deficiency in the different experimental groups, measurements of the urinary nicotinic acid, nicotinuric acid, trigonelline, and related compounds were carried out. The cyanogen bromide-p-aminoacetophenone method (Harris and Raymond, '39) was adapted for use with the Evelyn photoelectric colorimeter using a 440 filter. Each sample of urine was subjected first to three types of hydrolysis, according to the method of Melnick, Robinson and Field ('40). Cyanogen bromide was omitted from the blank since in our experience this procedure gave the most consistent values. The value for the sample subjected to ½ hour of acid (4N HCl) hydrolysis is thought to include all of the excreted nicotinic acid, nicotinamide, free pyridine, nicotine, approximately two-thirds of the nicotinuric acid, and any other unknown pyridine compounds that react with the reagents. The values obtained following 5-hour acid hydrolysis include, in addition to the above, all of the nicotinuric acid. Following alkaline (9N NaOH) hydrolysis of the urine for ½ hour, the value obtained is regarded as including all of the pyridine compounds determined by use of the prolonged acid hydrolysis plus about one-third of the voided trigonelline. By using conversion factors, it is possible to determine the total amount of trigonelline excreted; however, since these values are only semiquantitative, we have recorded only the actual values obtained through the chemical analyses. These data are summarized in table 3.

The urine samples were collected in two 24-hour periods and the sample for each period was analysed separately. All urine samples were collected under toluene. The tabulated results represent the average for the 2 days. The range of variation is also indicated. It is recognized that where the range of values is so wide, the average has little meaning. Averages have been recorded only to give some concept of the trend.

TABLE 3

Urinary excretion of nicotinic acid derivatives by pigs fed various experimental diets. All data are in milligrams per 24 hours (average and range).

	DIET AND SUPPLEMENTS						
	High protein plus				Low protein plus		Goldberger
HYDROLYSIS	TRN — 3[1] (10)[2]	TRChP — 4 (14)	TRB_6ChP — 5 (45)	$TRNB_6ChP$ — 6 (22)	TRB_6ChP — 7 (12)	$TRNB_6ChP$ — 8 (11)	TRB_6ChP — 9 (8)
½ hr. Acid (4 N HCl): "Nicotinic acid"	5.32 2.37– 7.40	1.87 0.–3.83	3.04 0.–11.96	3.82 0.56– 8.24	0.63 0.–3.18	1.63 0.– 6.85	1.56 0.– 7.14
5 hr. Acid (4 N HCl): "Nicotinuric acid"	5.23 2.36– 7.61	2.81 0.–5.40	4.15 0.–20.31	5.95 0.40–28.34	0.71 0.–2.35	2.43 0.– 7.17	3.37 0.–15.75
Alkaline (9 N NaOH): "Trigonelline"	6.06 1.35–12.19	2.65 0.–7.98	4.08 0.–22.92	4.75 0.57–16.27	1.08 0.–2.64	3.18 0.62–10.68	3.53 0.–12.70

[1] For meanings of these symbols see footnotes to table 1. The number signifies the number of the group.

[2] Numbers in parentheses indicate number of determinations.

In dogs fed a diet high in nicotinic acid, Sarett ('42) reported "nicotinic acid" urinary excretion values of 0.4 to 1.6 mg. and "trigonelline" of 1.5 to 7.3 mg. per 24 hours. In a dog during the onset of black tongue, the "nicotinic acid" was within the same range, but the "trigonelline" fell as low as 1.9 to 0.1 mg. Measurements of urinary "nicotinic acid" values in human subjects, both normal and deficient, have varied over a very wide range (Field et al., '41). In deficient subjects, Field and his associates ('41) noted an average, but not a constant, decrease in the urinary excretion of substances reacting like nicotinamide and a more marked and consistent decrease in the urinary excretion of trigonelline in the deficient subjects.

In our various experimental groups, there was a similar extremely wide range of variation in the excretion of nicotinic acid derivatives. One fact stands out, however. In the pigs fed the low protein diet without a supplement of nicotinic acid (group 7), (the only animals which showed marked evidence of nutritional deficiency somewhat resembling that ascribed to a lack of nicotinic acid), the excretion of nicotinic acid derivatives was very low and remained at a low level. Both the average and the maximum levels in this group were substantially lower than in any other experimental group. It is also possibly significant that, of all the groups of pigs receiving supplements of nicotinic acid, only those fed a low protein diet (group 8) at times excreted no "nicotinic" or "nicotinuric acid."

It is of interest that, in general, substantially lower values for the excretion of "trigonelline" were observed in all groups not receiving nicotinic acid as a supplement. The highest maximal values for "nicotinic acid" and "nicotinuric acid," however, were found in the pigs fed a high protein diet without nicotinic acid supplement (group 5) while the maximal "trigonelline" value in this group was only exceeded by that found in group 6 which was fed the same diet and received a supplement of nicotinic acid.

Najjar and Holt ('41) described the presence of certain fluorescent substances in the urine which they designated as F_1 and F_2. The latter is found in normal urine and tends to disappear in the presence of nicotinic acid deficiency. This substance (F_2) has been identified as N-methylnicotinamide (Huff and Perlzweig, '43). Subsequently Sarett ('43) showed that the so-called urinary "trigonelline" fraction is composed chiefly of N-methylnicotinamide although other methylated nicotinic acid derivatives are also included. Measurements of F_2 (N-methylnicotinamide) were made in a number of our pigs. The presence of this substance paralleled rather closely the excretion of the methylated

nicotinic acid derivatives ("trigonelline" fraction). In those animals receiving no nicotinic acid supplement, F_2 excretion was markedly diminished.[4]

DISCUSSION

Under the conditions of these experiments good growth and development occurred in pigs when they were given a diet containing a high percentage of a good grade of protein, supplemented with thiamine, riboflavin, pyridoxine, choline, and pantothenic acid, as well as vitamins A and D and sufficient quantities of fat, carbohydrate and minerals. This diet did not furnish significant quantities of nicotinic acid, even when "crude" casein was used. Microbiological assay (Snell and Wright, '41) of the casein used in most of our experiments showed it to contain less than 0.6 µg. nicotinic acid per gram.[5] Since our animals received at most 9.5 gm. casein per kilogram body weight, no more than 5.7 µg. of nicotinic acid per kilogram was derivable from this source. As a further check, however, two pigs were fed vitamin-free casein. These pigs also developed well. It is to be noted that these last two pigs received an experimental diet lacking in nicotinic acid from the ages of 16 and 22 days, respectively. Significant post-natal storage of nicotinic acid therefore, can scarcely be a factor.

The importance of nicotinic acid in nutrition was first demonstrated through the use of diets deficient in more than one respect. It was shown recently, however, that in the case of the dog loss of weight, inflammation of the gums, and reddening of the palate occur even when a highly purified diet similar to that used in our experiments and supplemented with the same five "B" vitamins is furnished (Schaefer et al., '42). Somewhat similar observations have been made in the chick (Briggs et al., '43). Experiments in monkeys (Harris, '38) and, as stated earlier, in pigs, were carried out by the use of diets of the Goldberger type and, therefore, cannot be regarded as having demonstrated a need for nicotinic acid as a supplement to a diet which is otherwise complete. A dietary source of nicotinic acid is not required by the rat (Dann, '41) or the sheep (Pearson et al., '40). Wooley and Sebrell ('45) have recently presented evidence that nicotinic acid is an essential growth factor for rabbits fed a purified diet.

Powick,[6] in studies which have been in progress for several years at the Beltsville Research Center of the U. S. Department of Agriculture,

[4] We are indebted to Dr. V. A. Najjar for these determinations.

[5] We are indebted to Dr. Daniel Melnick of the Food Research Laboratories, Inc., for this assay.

[6] Powick, W. C., personal communication.

has found impaired growth in a high proportion of young pigs fed a synthetic ration containing large amounts of acid-washed, alcohol-extracted casein (25%) and supplemented with thiamine, riboflavin, pyridoxine and pantothenic acid but devoid of nicotinic acid. It is of interest, in regard to the discrepancy between our observations and those of Powick, that Handler ('43), using a purified diet like that which Schaefer et al. ('42) used successfully in producing nicotinic acid deficiencies, could not induce a deficiency state in dogs which completely resembled that of black tongue dogs on Goldberger diets. Furthermore the weight loss, anorexia and other symptoms which did develop, disappeared spontaneously. Handler remarked that the inconsistent behavior of dogs on a purified ration made it difficult to evaluate the nature of the physiological defects in nicotinic acid deficiency.

Such observations, as well as our own, give ground for the belief that other factors as well as nicotinic acid play a role in the development of the deficiency syndrome which has been attributed to lack of this vitamin alone. According to the studies reported here, protein is one such factor. Only when the diet of our animals contained a small percentage of protein and lacked nicotinic acid as well (groups 7 and 7a), did signs of nutritional deficiency appear. These included marked impairment of growth, diarrhea, poor appetite, a rough coat, and anemia. The importance of the combined deficiency of protein and of nicotinic acid is indicated by the comparatively good condition of animals given diets deficient only in protein (group 8) or in nicotinic acid (group 5).[7]

The possibility that a deficiency of certain essential amino acids might be a factor in the etiology of pellagra was postulated as long ago as 1914 by Voegtlin ('19–'20). At that time, Goldberger and his coworkers ('15) showed that the development of the disorder could be prevented when the diet was supplemented with milk, fresh meat, eggs, and dried beans. The classical diet which has been used in the experimental production of canine black tongue is poor in protein as well as in B vitamins.

It is recognized that the pyridine nucleotides play a role in protein metabolism (Ball, '39). Handler and Dann ('42) reported that nicotinamide inhibits the growth of rats, probably due to the deprivation of the animal's supply of methyl groups because of trigonelline synthesis. The inhibition of growth could be prevented by the administration of

[7] The view here expressed that the quantity of protein and perhaps, in particular, of certain essential amino acids in the diet, is important as affecting nicotinic acid requirement, finds further support in the recent observation by Krehl, Tepley, Sarma and Elvehjem ('45, Science, vol. 101, p. 489). These workers present evidence "that protein, or tryptophane, particularly, may have a profound effect on the nicotinic acid requirement."

methionine and by choline plus homocystine. Sarett, Klein and Perlzweig ('42) noted a decreased urinary excretion of nicotinic acid in dogs when the protein content of the diet was increased, and an increased excretion when the protein content was lowered. Their observation in the dog agrees with ours in the pig only in that an inter-relationship of protein and nicotinic acid is suggested; somewhat at variance is our finding that the excretion of nicotinic acid derivatives was lowest in pigs receiving a low protein diet not supplemented with nicotinic acid. The experiment of Sarett, Klein and Perlzweig did not include a group fed a diet deficient in both protein and nicotinic acid. Sarett and Perlzweig ('43) later reported that the nicotinic acid content of the livers of rats fed a low protein diet was low as compared with the livers of those fed high protein diets, whether the vitamin intake was high or low. Differences in species and in experimental methods make further comparisons of their observations and ours impossible.

None of these observations regarding the relationship of protein and nicotinic acid, however, serve to explain the dramatic improvement noted in two pigs by Chick and her co-workers ('38) when only nicotinic acid was added to the "Goldberger maize diet" which her animals were being fed. Nor do they explain the observations of other investigators (Hughes, '38; Madison et al., '39; Wintrobe, '39) which led to the conclusion that a dietary source of nicotinic acid is needed by the pig. Since the diets furnished in the above experiments were probably lacking in other B vitamins besides nicotinic acid, our findings would seem to indicate that in some way the pig is able to make up for a lack of nicotinic acid in the diet when thiamine, riboflavin, pyridoxine, choline, and pantothenic acid are furnished. Perhaps the presence of these vitamins is beneficial by making possible the bacterial synthesis of nicotinic acid in the intestines, or by synthesis of the vitamin in the body. Our attempt to show that synthesis of nicotinic acid was mediated in some way by pyridoxine, which was the reason for our group 4, gave inconclusive results. It is to be noted that nicotinic acid and pantothenic acid are closely related both in chemical properties and in distribution (Elvehjem, '40). Study of the biological interrelationship of these two vitamins would seem to be indicated. We have described elsewhere (Wintrobe, Follis, Alcayaga et al., '43) the severe colitis which develops in pigs deficient in pantothenic acid and have pointed out the similarity of these changes in the bowel to those which have been described under such titles as "necrotic enteritis" and "pig pellagra," and have been attributed to nicotinic acid deficiency.

Various investigators (Miller and Rhoads, '35; György, '38; Day et al., '40; Handler and Featherston, '40) have described anemia and leukopenia in various animals receiving black tongue-producing diets or diets lacking nicotinic acid. In our animals leukopenia was never noted. When nicotinic acid alone was lacking, no anemia or other morphological abnormalities were found, but when the diet was also poor in protein, severe normocytic anemia developed. Since severe infections occurred in these animals, no conclusions can be drawn regarding the significance of this anemia.

The measurement of the urinary excretion of nicotinic acid derivatives has not proved to be a wholly satisfactory method for assessing nicotinic acid deficiency (Perlzweig et al., '42). Nevertheless, it is of interest that a correlation between such excretion and the presence of signs of nutritional deficiency was observed in the pigs fed the low protein diet without nicotinic acid supplement.

The development of chromatolysis in the dorsal root ganglion cells of four out of five of the pigs receiving the low protein-nicotinic acid deficient diet (groups 7 and 7a) is interesting but its significance is not entirely clear.

SUMMARY

1. Observations in sixty-one pigs are described in which the effects of low and high protein diets supplemented with thiamine, riboflavin, pyridoxine, choline, pantothenic acid, inositol, and p-aminobenzoic acid, with and without nicotinic acid, are compared.

2. Young pigs fed a high protein (26.1% casein) diet supplemented with the first five vitamins named above, and not furnished with nicotinic acid, showed no signs of nutritional deficiency except for slightly less satisfactory growth in certain instances.

3. When the protein content of the diet was low (10% casein) the omission of nicotinic acid from the diet was associated with the development of signs of nutritional deficiency (markedly impaired growth, rough coats, diarrhea, poor appetite and severe anemia).

4. Only in the last mentioned pigs was there a marked and consistent reduction in the urinary excretion of nicotinic acid derivatives.

5. There appears to be a close nutritional relationship between protein and nicotinic acid.

ACKNOWLEDGMENTS

Vitamins used in these experiments were furnished by Merck and Company, Inc., and yeast and cod liver oil by Mead, Johnson and Company.

Technical aid was given by Mr. Adolph Suksta, Miss Gertrude Merr, and Mrs. Eleanor Milnor Collins.

LITERATURE CITED

BALL, E. G. 1939 Chemical reactions of nicotinic acid amide in vivo. Bull. Johns Hopkins Hosp., vol. 65, p. 253.

BRIGGS, G. M., JR., T. D. LUCKEY, L. J. TEPLY, C. A. ELVEHJEM AND E. B. HART 1943 Studies on nicotinic acid deficiency in the chick. J. Biol. Chem., vol. 148, p. 517.

CHICK, HARRIETTE, T. F. MACREA, A. J. P. MARTIN AND C. J. MARTIN 1938 III. Curative action of nicotinic acid on pigs suffering from the effects of a diet consisting largely of maize. Biochem. J., vol. 32, p. 10.

DANN, W. J. 1941 The synthesis of nicotinic acid by the rat. J. Biol. Chem., vol. 141, p. 803.

DAY, P. L., W. C. LANGSTON, W. J. DARBY, J. G. WAHLIN AND V. MIMS 1940 Nutritional cytopenia in monkeys receiving the Goldberger diet. J. Exp. Med., vol. 72, p. 463.

ELVEHJEM, C. A. 1940 Relation of nicotinic acid to pellagra. Physiol. Rev., vol. 20, p. 249.

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOLLEY 1938 The isolation and identification of the anti-black tongue factor. J. Biol. Chem., vol. 123, p. 137.

FIELD, H., JR., D. MELNICK, W. D. ROBINSON AND C. F. WILKINSON, JR. 1941 Studies on the chemical diagnosis of pellagra (nicotinic acid deficiency). J. Clin. Inv., vol. 20, p. 379.

GOLDBERGER, J., C. H. WARING AND D. G. WILLETS 1915 The prevention of pellagra. A test of diet among institutional inmates. Pub. Health Rep.. vol. 30, p. 3117.

GYÖRGY, P. 1938 Nicotinic acid and prevention of nutritional panmyelophthisis in rats. Proc. Soc. Exp. Biol. Med., vol. 37, p. 732.

HANDLER, P. 1943 Use of highly purified rations in the study of nicotinic acid deficiency. Proc. Soc. Exp. Biol. Med., vol. 52, p. 263.

HANDLER, P., AND W. J. DANN 1942 The inhibition of rat growth by nicotinamide. J. Biol. Chem., vol. 146, p. 357.

HANDLER, P., AND W. P. FEATHERSTON 1943 The biochemical defect in nicotinic acid deficiency. II. On the nature of the anemia. J. Biol. Chem., vol. 151, p. 395.

HARRIS, L. J. 1938 The vitamin B_2 complex. VII. Further notes on "monkey pellagra" and its cure with nicotinic acid. Biochem. J., vol. 32, p. 1479.

HARRIS, L. J., AND W. D. RAYMOND 1939 Assessment of the level of nutrition. A method for the estimation of nicotinic acid in urine. Biochem. J., vol. 33, p. 2037.

HUFF, JESSE W., AND W. A. PERLZWEIG 1943 N-methylnicotinamide, a metabolite of nicotinic acid in urine. J. Biol. Chem., vol. 150, p. 395.

HUGHES, E. H. 1938 The vitamin B-complex as related to growth and metabolism in the pig. Hilgardia, vol. 11, p. 10.

MADISON, L. C., R. C. MILLER AND T. B. KEITH 1939 Nicotinic acid in swine nutrition. Science, vol. 89, p. 490.

MELNICK, D., W. D. ROBINSON AND H. FIELD, JR. 1940 Influence of the excretion of other pyridine compounds upon the interpretation of the urinary nicotinic acid values. J. Biol. Chem., vol. 136, p. 131.

MILLER, D. K., AND C. P. RHOADS 1935 Experimental production of loss of hematopoietic elements of gastric secretion and of liver in swine with achlorhydria and anemia. J. Clin. Inv., vol. 14, p. 153.

NAJJAR, V. A., AND L. E. HOLT, JR. 1941 The excretion of specific fluorescent substances in the urine in pellagra. Science, vol. 93, p. 20.

PEARSON, R. B., A. H. WINEGAR AND H. SCHMIDT 1940 Studies on the metabolism of nicotinic acid in the sheep. J. Nutrition, vol. 20, p. 551.

PERLZWEIG, W. A., H. P. SARETT AND L. H. MARGOLIS 1942 Studies in nicotinic acid metabolism. V. A test for nicotinic acid deficiency in man. J. Am. Med. Assn., vol. 118, p. 28.

SARETT, H. P. 1942 Studies in nicotinic acid metabolism. II. The fate of nicotinic acid in normal and black tongue dogs. J. Nutrition, vol. 23, p. 35.

——— 1943 A direct method for the determination of N-methyl derivatives of nicotinic acid in urine. J. Biol. Chem., vol. 150, p. 159.

SARETT, H. P., J. R. KLEIN AND W. A. PERLZWEIG 1942 The effect of the level of protein intake upon the urinary excretion of riboflavin and nicotinic acid in dogs and rats. J. Nutrition, vol. 24, p. 295.

SARETT, H. P., AND W. A. PERLZWEIG 1943 The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats. J. Nutrition, vol. 25, p. 173.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Nicotinic acid deficiency studies in dogs. J. Biol. Chem., vol. 144, p. 679.

SNELL, E. E., AND L. D. WRIGHT 1941 Microbiological method for determination of nicotinic acid. J. Biol. Chem., vol. 139, p. 675.

VOEGTLIN, C. 1919–1920 Recent work on pellagra. The Harvey Lectures, vol. 15, p. 87.

WAISMAN, H. A., AND C. A. ELVEHJEM 1940 Multiple deficiencies in the modified Goldberger diet as demonstrated with chicks. J. Nutrition, vol. 20, p. 519.

WINTROBE, M. M. 1939 Nutritive requirements of young pigs. Am. J. Physiol., vol. 126, p. 375.

WINTROBE, M. M., R. H. FOLLIS, JR., R. ALCAYAGA, M. PAULSON AND S. HUMPHREYS 1943 Pantothenic acid deficiency in swine. With particular reference to the effects on growth and on the alimentary tract. Bull. Johns Hopkins Hosp., vol. 73, p. 313.

WINTROBE, M. M., R. H. FOLLIS, JR., M. H. MILLER, H. J. STEIN, R. ALCAYAGA, S. HUMPHREYS, A. SUKSTA AND G. E. CARTWRIGHT 1943 Pyridoxine deficiency in swine. With particular reference to anemia, epileptiform convulsions and fatty liver. Bull. Johns Hopkins Hosp., vol. 72, p. 1.

WINTROBE, M. M., M. H. MILLER, R. H. FOLLIS, JR., H. J. STEIN, C. MUSHATT AND S. HUMPHREYS 1942 Sensory neuron degeneration in pigs. IV. Protection afforded by calcium pantothenate and pyridoxine. J. Nutrition, vol. 24, p. 345.

WINTROBE, M. M., J. L. MILLER, JR. AND H. LISCO 1940 The relation of diet to the occurrence of ataxia and degeneration in the nervous system of pigs. Bull. Johns Hopkins Hosp., vol. 67, p. 377.

WINTROBE, M. M., D. M. MITCHELL AND L. C. KOLB 1938 Sensory neuron degeneration in vitamin deficiency. Degeneration of the posterior columns of the spinal cord, peripheral nerves, and dorsal root ganglion cells in young pigs fed a diet containing thiamine (B_1) and riboflavin but otherwise deficient in vitamin B complex. J. Exp. Med., vol. 68, p. 207.

WOOLEY, J. S., AND W. H. SEBRELL 1945 Niacin (nicotinic acid), an essential growth factor for rabbits fed a purified diet. J. Nutrition, vol. 29, p. 191.

THE THERAPEUTIC EFFECT OF YEAST AND PYRIDOXINE ON POIKILOCYTOSIS IN DAIRY CATTLE [1]

J. T. REID,[2] C. F. HUFFMAN AND C. W. DUNCAN

Institute of Nutrition and the Departments of Dairy and Agricultural Chemistry, Michigan State College, East Lansing

(Received for publication July 12, 1945)

The work of several investigators has suggested a relationship of nutrition to the occurrence of poikilocytosis in animals. The study reported in this paper was initiated for the purpose of investigating the relationship of anti-anemia mineral mixtures, yeast, pyridoxine, riboflavin and nicotinic acid to the alleviation of a poikilocytic condition found to be prevalent in some farm herds and produced experimentally in young calves by feeding semi-restricted rations. The results are of additional interest because of the absence in the literature of specific data on the production of poikilocytosis in dairy animals.

Poikilocytosis has been reported in relation to scurvy and anemia studies in guinea pigs (Hanke and Koessler, '28), anemia in suckling pigs (Kernkamp, '32), pigeons fed a vitamin B-complex deficient diet (Hogan et al., '40), anemic calves (Knoop et al., '35) and cobalt-deficient calves (Neal and Ahmann, '37).

In a recent study Reid, Huffman and Duncan ('43, '45) found a high incidence of poikilocytosis in Michigan dairy cattle accompanied by anorexia, retarded growth and general unthriftiness. Some herds manifested poikilocytosis while neighboring herds were completely unaffected. Many of the affected animals were anemic while others were normal in this respect. Since the kind and quality of rations fed to these animals varied greatly, it was believed that the condition was associated with a nutritional deficiency.

EXPERIMENTAL

Most of the data reported in this paper were derived from animals receiving a whole milk and cereal breakfast food [3] ration although many

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article no. 768 (n.s.).

[2] From a thesis presented to the Graduate School of Michigan State College in partial fulfillment for the Ph.D. degree.

[3] Rice Krispies.

other calves received the individual farm ration. Some calves were treated with only one supplement, others were treated with several supplements simultaneously, while others received several supplements for varying periods but only one supplement at a time.

Two mineral mixtures, no. 1 and no. 2, were used. The no. 1 mixture supplemented the ration of seven affected calves with 250 mg. of iron, 250 mg. of manganese and 25 mg. of copper daily, while the no. 2 mixture furnished 5 mg. of cobalt daily to twenty affected calves in addition to the minerals supplied in the no. 1 mixture.

Two calves received 200 gm. of dry brewers' yeast per day while a third calf received 200 gm. and later 450 gm. of the same yeast per day. Two calves received 100 gm. of live yeast daily in addition to the no. 2 mineral mixture. Several of the B-complex vitamins were administered to ascertain the anti-poikilocytic factor(s) provided by yeast. One calf received 50 mg. of nicotinic acid per day and another calf received 25 mg. of riboflavin per day. These vitamins were administered for a period of 70 days after which time they were replaced with 40 mg. of pyridoxine daily. Pyridoxine [4] was fed to eight other calves in amounts ranging from 5 to 40 mg. per day. Some of the calves also received the no. 1 or no. 2 mineral supplement at the same time.

The microscopic examination of the red blood cells of four rumen fistula cows, all having open or unplugged fistulas, revealed the presence of from 15 to 99% poikilocytes. The condition of the most severely affected rumen fistula cow was corrected on two occasions by feeding brewers' yeast. The poikilocytic condition reoccurred, however, sometime after the yeast treatment was terminated.

The progress of recovery of the affected animals was followed by the microscopic examination of blood smears and occasionally by hanging drop preparations. Weight gains were used also as a criterion of the animal's general status of health. The hemoglobin, red blood cell count, red cell volume, and the plasma calcium, inorganic phosphorus and magnesium concentrations were determined by methods previously reported (Reid et al., '45).

Forty-two calves and four rumen fistula cows were employed in this study.

RESULTS

The results obtained from feeding the various supplementary factors in addition to the regular ration of the calves are shown in tables 1–6.

[4] The authors wish to thank Merck and Co., Inc., for furnishing a portion of the pyridoxine used.

Many animals were encountered during the course of this investigation that showed a typical anemia blood picture uncomplicated with poikilocytosis. The data obtained from calf A in table 1 illustrate a case of this type. Although the blood of most of the poikilocytosis affected animals revealed low hemoglobin concentrations, some of the animals were normal in this respect. The data for B in table 1 show a severely affected animal whose hemoglobin level is nearly normal. The data also show the loss in weight accompanying the course of a typical case of poikilocytosis when no therapeutic agent was administered.

The values included in several tables for plasma calcium, inorganic phosphorus and magnesium indicate no relationship of these constituents to the poikilocytosis anomaly.

TABLE 1

Blood picture of a calf (A) showing oligochromemia but unaffected by poikilocytosis and a calf (B) affected with poikilocytosis but not showing oligochromemia.

CALF	AGE	BODY WT.	HB	R.B.C.V.	R.B.C.C.	POIKILOCYTES	PLASMA Ca	PLASMA Inorg. P	PLASMA Mg
no.	*days*	*kg.*	*gm. %*	*%*	*mill./mm.³*	*%*	*mg. per 100 ml.*		
A	240	103.2	6.7	18.0	5.40	0	9.9	7.02	1.24
	250	102.6	6.5	18.0	5.07	0	10.2	5.34	1.30
	260	112.5	8.0	23.0	3.97	0	11.6	7.86	1.98
	270	115.7	8.0	23.0	5.55	0	11.0	6.51	1.72
	280	116.4	9.5	24.7	6.93	0	10.9	5.70	1.57
B	80	36.7	10.9	.	...	90	11.1	6.32	1.87
	90	33.0	10.9	.	..	90	8.8	6.32	0.99
	100	33.4	10.9		.	95	10.2	5.34	1.03
	110	30.2	11.3	..	...	90	11.0	5.90	1.03
	120	30.0	10.3	. .	. .	94–97	11.1	4.96	1.01

Effect of mineral supplementation on poikilocytosis. Table 4 shows the effect of mineral supplementation of the ration of a representative animal. These data show a gradual increase in the hemoglobin concentration without any change in the severity of poikilocytosis.

Treatment with dry brewers' yeast. Table 2 shows the effectiveness of brewers' yeast in the treatment of poikilocytosis in two calves. Since these calves exhibited symptoms similar to those observed in cobalt deficiency, this element was administered for 7 weeks without response prior to the feeding of yeast. (The symptoms of cobalt deficiency disease are always alleviated within a week after the initial treatment.) The cobalt supplement was then discontinued and 200 gm. of brewers' yeast was fed daily. The distortion of the red blood cells of the calves was markedly ameliorated although no change in the level of hemoglobin

was observed. From the percentage of poikilocytes recorded in the table it appears that the distorted red cells can be restored to normally shaped corpuscles by feeding dry brewers' yeast for 3 to 4 weeks. The data indicate also the stunted condition of the calves prior to the feeding of yeast. An increased appetite and an impetus in weight gained was observed as poikilocytosis subsided. Three calves received the yeast supplement and all responded in the same manner. These findings are similar to those reported by Hogan et al. ('40) in that poikilocytosis in pigeons was corrected after 5 weeks of yeast supplementations.

TABLE 2

Effect of adding brewers' yeast to the ration.

CALF	DATE	AGE	BODY WT.	POIKILOCYTES	HB	CALF	DATE	AGE	BODY WT.	POIKILOCYTES	HB
no.		*days*	*kg.*	*%*	*gm. %*	*no.*		*days*	*kg.*	*%*	*gm. %*
C-3	9/24	348		75	9.8	C-4	9/24	237	. . .	25	11.6
	10/15	369	142.2	80	9.1		10/15	258	175.3	20	11.7
	11/13 [1]	397	146.3	75–80	9.1		11/13 [1]	286	179.8	23–25	11.9
	11/28	412	142.2	50–75	8.8		11/28	301	197.5	10–15	11.2
	12/18	432	165.3	5	9.6		12/18	321	202.1	0	11.7

[1] Calf received 200 gm. of brewers' yeast daily after this date.

TABLE 3

Data obtained from a representative poikilocytosis affected animal treated with live yeast.

AGE	BODY WT.	DAILY INTAKE PER KG. BODY WT.				HB	POIKILOCYTES	PLASMA		
		Fe	Cu	Mn	Co			Ca	Inorg. P	Mg
days	*kg.*	*mg.*	*μg.*	*mg.*	*μg.*	*gm. %*	*%*	*mg. per 100 ml.*		
10	36.7	0.25	43.7	13.2	3.8	6.3	75	11.5	6.19	1.84
15		Added 100 gm. live yeast and no. 2 mineral mixture daily								
15	38.9	8.86	711.6	16.2	137.7	5.7	70–75	11.2	7.10	1.69
20	41.1	8.39	673.8	18.0	147.6	7.3	20–25	11.0	7.02	2.13
30	46.2	7.47	601.0	16.6	123.0	8.2	4	11.7	4.19	1.93
40	50.5	6.86	554.5	13.7	113.0	9.8	3	11.3	9.06	1.88
50	53.6	7.79	539.7	15.6	111.6	10.7	1	11.2	7.96	1.99
60	61.4	6.91	473.6	15.5	98.1	12.6	0	11.1	8.12	1.72

Treatment with live yeast. The effect of live yeast in correcting the deformed red cell condition in calves is shown in table 3. These data show the correction of the poikilocytic condition to nearly normal-shaped erythrocytes after a 4-week period during which time the calf consumed 100 gm. of live yeast daily. A remarkable decrease in the number of poikilocytes was noted after the first and second week of yeast feeding as well as a striking transformation in the shape of the poikilocytes —

the degree of distortion became less extreme. An increased growth rate accompanied the disappearance of the poikilocytes in the blood. Only two calves were treated with live yeast but the results were uniformly the same. Since both of the calves had received the no. 2 mineral mixture simultaneously with the live yeast, it was necessary to eliminate one or the other of the supplements in order to derive the correction factor. The correction of the poikilocytic condition is attributed entirely to the ingested yeast since the mineral supplements failed to alter the extreme poikilocytosis of animals for which the data in table 4 are representative.

TABLE 4

Data obtained from a representative poikilocytosis affected animal treated with pyridoxine.

AGE [1]	BODY WT.	DAILY INTAKE PER KG. BODY WT.				HB	POIKILO-CYTES	PLASMA		
		Fe	Cu	Mn	Co			Ca	Inorg. P	Mg
days	*kg.*	*mg.*	*µg.*	*mg.*	*µg.*	*gm. %*	*%*	*mg. per 100 ml.*		
50	67.3	2.52	60.4	12.0	11.9	9.0	85	11.8	6.44	2.40
70	69.8	1.29	42.6	9.9	6.9	7.0	85–90	11.9	8.56	1.18
72	Added no. 1 mineral mixture									
83	75.9	5.97	392.6	9.3	12.5	9.3	90	11.2	6.69	0.99
83	Discontinued no. 1 mineral mixture and added no. 2 mineral mixture									
100	80.1	6.26	380.6	8.9	63.9	9.5	90	11.7	5.76	1.10
120	86.4	5.09	346.3	9.3	68.5	8.9	90	11.7	6.01	1.01
137	99.1	5.73	313.7	8.1	50.9	11.6	90	11.9	7.35	2.09
137	Added 40 mg. pyridoxine daily									
140	101.6	5.73	313.7	8.1	50.9	11.6	85	11.9	7.35	2.09
150	118.9	4.21	308.6	8.0	50.0	13.3	40	11.8	6.95	1.28
160	126.8	4.03	307.3	8.5	49.8	11.3	1–5	12.2	8.56	1.14
168	134.4	3.85	300.1	7.9	47.1	10.7	0	12.2	5.74	1.03

[1] Data were obtained at 7-day intervals and on days when supplements were changed.

Treatment with pyridoxine. Eight animals varying in the severity of poikilocytosis were fed different amounts of pyridoxine. Although the daily ingestion of 5, 10, 15 or 20 mg. of pyridoxine was effective in ameliorating the poikilocytic condition, the recovery was slow. Forty milligrams of pyridoxine daily, however, resulted in the complete absence of poikilocytes within 3 or 4 weeks after treatment was begun. Tables 4–6 show the rapidity with which the poikilocytes disappeared from the blood. The disappearance of the poikilocytes was accompanied by an improvement in the general health of the animals and was also reflected by an accelerated growth rate.

Table 5 shows the ineffectiveness of feeding 25 mg. of riboflavin per day on the poikilocytosis of a representative animal. After a 70-day period, riboflavin feeding was discontinued, and 40 mg. of pyridoxine was added to the animal's ration. Although riboflavin treatment did

TABLE 5

Effects of riboflavin and pyridoxine on poikilocytosis.

DATE [1]	BODY WT.	HB	R.B.C.C.	R.B.C.V.	POIKILO-CYTES	PLASMA Ca	PLASMA Inorg. P	PLASMA Mg
	kg.	*gm. %*	*mill./mm.*^{3}	*%*	*%*	*mg. per 100 ml.*		
4/11	48.2	8.7	9.70	25.0	5	11.3	6.87	1.65
5/9	58.2	8.7	10.72	28.0	30	12.1	8.39	1.64
5/15			Added 100 mg. Fe daily					
6/6	75.0	12.5	13.96	44.0	40	9.8	9.06	2.20
6/14			Discontinued Fe					
6/14	75.0	11.9	12.15	32.0	40	11.7	9.47	2.11
7/11	84.5	10.1	10.27	27.0	45–50	11.4	6.79	2.08
8/8	96.8	8.7	8.21	25.5	55	12.5	7.44	2.06
8/29	105.5	9.1	7.27	28.0	50	12.3	8.56	2.06
9/5			Added 25 mg. of riboflavin daily					
9/5	108.2	7.3	7.05	21.0	40	11.1	5.63	1.63
10/6	138.1	9.1	11.60	26.5	40	10.5	9.06	1.54
10/24	143.1	9.5		30.0	45	10.9	8.12	1.18
11/7	152.7	9.5	9.55	27.0	45	11.5	6.72	1.16
11/13	168.6	Discontinued riboflavin and added 40 mg. pyridoxine daily						
11/15	170.5	9.2	13.04	26.5	40	11.3	7.67	1.73
11/22	176.8	8.3	9.65	25.0	15	11.5	9.33	2.08
11/27	181.8	8.6	6.70	23.5	5–7	10.8	7.91	1.68
12/5	186.8	8.8	7.65	25.0	1	10.8	7.44	1.96
12/11	189.6	9.4	8.31	26.0	0	11.5	6.76	1.87

[1] Data were obtained at 7-day intervals and on days when supplements were changed.

TABLE 6

Effects of nicotinic acid and pyridoxine on poikilocytosis.

DATE [1]	BODY WT.	HB	R.B.C.C.	R.B.C.V.	POIKILO-CYTES	PLASMA Ca	PLASMA Inorg. P	PLASMA Mg
	kg.	*gm. %*	*mill./mm.*^{3}	*%*	*%*	*mg. per 100 ml.*		
8/8	153.9	9.0	9.06	26.0	35	12.5	6.44	2.33
8/29	164.5	10.4	8.38	31.0	35	12.1	9.19	1.76
9/5			Added 50 mg. nicotinic acid daily					
9/5	171.5	9.8		26.5	30	11.3	7.86	2.10
10/6	187.5	9.8	7.24	29.5	30–35	11.1	7.67	1.73
10/24	195.0	9.0		28.0	35	10.9	7.14	2.11
11/7	205.1	9.4	6.95	26.0	45	11.2	6.57	1.37
11/13	213.7	Discontinued nicotinic acid and added 40 mg. pyridoxine daily						
11/15	218.5	9.0	6.50	26.0	40	11.4	6.79	1.35
11/22	221.6	8.7	8.05	24.0	15	11.2	6.87	2.05
11/27	226.6	8.7	5.81	24.5	7	11.2	5.19	2.15
12/5	231.48	9.1	7.86	25.5	1	9.9	4.77	2.02
12/11	241.36	9.1	7.15	25.0	0	10.9	6.51	2.19

[1] Data were obtained at 7-day intervals and on days when supplements were changed.

not affect the number of poikilocytes, the pyridoxine treatment corrected the condition to normal.

Table 6 shows that 50 mg. of nicotinic acid daily for 70 days failed to correct poikilocytosis in a representative animal, whereas the transformation to normal was attained after treatment with 40 mg. of pyridoxine daily. It should be emphasized that the decreased rate of growth and the poikilocytic status of the animals remained unchanged during the mineral supplement, riboflavin and nicotinic acid feeding periods, but rapid response was obtained when pyridoxine was added to the ration.

DISCUSSION

Different agents known to stimulate hemapoiesis were fed in an effort to correct the poikilocytic status of calves and rumen fistula cows. The mineral supplements had no effect on poikilocytosis although a noticeable elevation in the hemoglobin level resulted from their supplementation. The feeding of either brewers' yeast or live yeast was effective in ameliorating the poikilocytic condition within 3 to 4 weeks.

Several of the B-complex vitamins contained in yeast were used to treat the poikilocytic animals in an attempt to derive the curative factor(s) for this anomaly. The use of riboflavin and nicotinic acid did not alter the shape of the poikilocytes but pyridoxine administration resulted in a specific response. In cases where severe poikilocytosis and retarded growth were found, the ingestion of pyridoxine resulted in an increase in appetite and an accelerated growth rate coincident with the subsidence of the poikilocytes. In the less severe cases the increase in the rate of growth after the administration of pyridoxine was not appreciable. Although the daily ingestion of 5 to 20 mg. of pyridoxine brought about slow recovery, the ingestion of 40 mg. accelerated the complete restoration of the poikilocytes to normal erythrocytes. Recovery was effected within 3 to 4 weeks. The prognosis of the animals treated with 40 mg. of pyridoxine was excellent and the cure was permanent.

In most of the cases observed, young calves were the animals most commonly affected with poikilocytosis whereas the incidence in mature animals was quite limited. Observation was made also of the apparently spontaneous recovery of previously affected calves. It seems logical to suppose that recovery occurred concomitant with the development of the rumen flora and fauna.

Since the blood of four rumen fistula cows revealed the presence of poikilocytes, the existence of the open rumen fistulas offered the possi-

bility that a derangement in ruminal function may have been responsible for the occurrence of the poikilocytes. Since the young calves receiving a restricted ration of whole milk and a cereal breakfast food generally manifested poikilocytosis and the older animals did not, it appeared that a deficiency of some factor(s) necessary for the maintenance of corpuscular integrity may be synthesized in the normal mature rumen. The early studies of Theiler et al. ('15) and Bechdel et al. ('26, '27) demonstrated that cattle were healthy and normal on vitamin B-complex deficient rations, while rumen synthesis of the complex was reported by Scheunert and Schieblich ('23), Bechdel et al. ('28) and Wegner and coworkers ('40, '41). The latter group of investigators believed that the increase in B-vitamins in the rumen ingesta, as compared to the ration fed, was due to synthesis and not to a concentration effect. Arnold and Elvehjem ('40) stated that ruminant animals are able to satisfy their requirements for at least some members of the B-complex by virtue of the fermentation which occurs in the digestive tract. The synthesis of pyridoxine by the bacteria in the rumen has been pointed out by several workers (McElroy and Goss, '40). Wegner and coworkers ('40, '41) showed that the pyridoxine content of the rumen ingesta is higher than that of the ration fed and concluded that the increase was due to bacterial synthesis. These findings support the earlier work of McElroy and Goss ('40) in that the vitamin B_6 potency of the rumen contents is 6 to 8 times as great as that found in the ration. These investigators favored a true synthesis of the vitamin related to growth of the rumen flora rather than a concentration caused by differential rates of passage of different nutrients from the rumen. No mention was made as to whether the rumen fistulas were open or closed. They showed that a normal amount of vitamin B_6 is found in the milk of a cow on a vitamin B_6 deficient ration which seems to indicate that the cow was able to synthesize enough of this vitamin to satisfy her maintenance needs and in addition transfer a normal amount into the milk.

No reports concerning the rumen synthesis of pyridoxine have indicated that the rumen of young calves has flora developed sufficiently to synthesize this vitamin in quantity large enough to satisfy the animal's maintenance requirements or that the vitamin is synthesized at all. It seems logical, therefore, to suppose that the ration fed, the age of the calf, and the condition of the rumen of both rumen fistula cows and normal calves are factors concerned in the synthesis of pyridoxine.

Although no known case of vitamin B_6 deficiency in cattle has been reported, a deficiency of this vitamin has been reported in other animals.

Fouts and associates ('38) successfully treated a severe microcytic hypochromic anemia in dogs with vitamin B_6 after iron therapy had failed to relieve the anemia. Later Fouts et al. ('40) showed that adult dogs as well as puppies developed vitamin B_6 deficiency anemia which did not respond to iron and copper therapy. McKibbin and coworkers ('39) reported that the ingestion of a low level of vitamin B_6 is necessary for growth but the ingestion of a higher level is necessary to protect pyridoxine deficient dogs against hypochromic microcytic anemia. They suggested 60 µg. of vitamin B_6 as an anemia-curative dose for dogs.

The ability of pyridoxine to stimulate the hemapoietic system of deficient patients has been extensively demonstrated by increased blood regeneration and the restoration of the red corpuscles to normal size. The vitamin was used by Vilter and associates ('40) to treat three pellagrins with macrocytic anemia and two patients with pernicious anemia. A subjective relief and a slight reticulocytosis occurred. Although these investigators stated that vitamin B_6 definitely affects the hemapoietic system of humans who have the macrocytic anemia of pellagra or pernicious anemia, they did not imply that it is an antipernicious anemia factor. It should be emphasized, however, that pernicious anemia is one of the diseases constantly associated with poikilocytosis. Wintrobe et al. ('43) produced a severe anemia in pigs by feeding a pyridoxine deficient diet. When pyridoxine was administered, a rapid regeneration of blood accompanied the restoration of normal corpuscular size. The reports in the literature indicate that simple stomach animals and humans may suffer from a lack of vitamin B_6 whereas a deficiency of this vitamin in the normal ruminant is unlikely to occur by virtue of bacterial synthesis. Poikilocytosis, however, has not been associated with pyridoxine deficiency in simple stomach animals.

This investigation has shown that when poikilocytes do occur in the blood of calves they can be replaced with normal erythrocytes following the administration of brewers' yeast, live yeast or vitamin B_6. The results of this study have indicated also the possibility of a lack of or a derangement of the normal rumen activity necessary for the synthesis or utilization of pyridoxine either for the direct maintenance of corpuscular integrity or for the factor(s) responsible for this function.

SUMMARY

Young calves on semi-restricted rations and cows with large open fistulas of the rumen were found to be the dairy animals most commonly affected with poikilocytosis. Dry brewers' yeast, live yeast, or pyridoxine were effective therapeutic agents for the treatment of this condi-

tion, whereas the ingestion of nicotinic acid, riboflavin or a mineral mixture containing iron, copper, cobalt and manganese did not elicit any curative effects on the disease.

The occurrence and the degree of severity of poikilocytosis were independent of the hemoglobin content, red blood cell count, red blood cell volume and the plasma concentration of calcium, inorganic phosphorus and magnesium.

As a result of this investigation the hypothesis is offered that the primary defect responsible for the occurrence of poikilocytosis in dairy animals is due to a lack of or an interference with normal ruminal activity.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1940 The relation of the vitamin B complex to the nutrition of domestic animals. J. Am. Med. Assn., vol. 96, p. 56.

BECHDEL, S. I., C. H. ECKLES AND L. S. PALMER 1926 Vitamin B requirement of the calf. J. Dairy Sci., vol. 9, p. 409.

BECHDEL, S. I., AND H. E. HONEYWELL 1927 The relation between the vitamin B contents of the feed eaten and of the milk produced. J. Agric. Research, vol. 35, p. 283.

BECHDEL, S. I., H. E. HONEYWELL, R. A. DUTCHER AND M. H. KNUTSEN 1928 Synthesis of vitamin B in the rumen of the cow. J. Biol. Chem., vol. 80, p. 231.

FOUTS, P. J., O. M. HELMER AND S. LEPKOVSKY 1940 Nutritional microcytic hypochromic anemia in dogs cured with crystalline factor I. Am. J. Med. Sci., vol. 199, p. 163.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES 1938 Production of microcytic hypochromic anemia in puppies on synthetic diet deficient in rat antidermatitis factor (vitamin B_6). J. Nutrition, vol. 16, p. 197.

HANKE, M. T., AND K. K. KOESSLER 1928 The effect of scurvy-producing diets and tyramine on the blood of guinea pigs. J. Biol. Chem., vol. 80, p. 499.

HOGAN, A. G., L. R. RICHARDSON, P. E. JOHNSON AND R. N. NISBET 1940 Pigeon anemia as a deficiency disease. J. Nutrition, vol. 20, p. 203.

KERNKAMP, H. C. H. 1932 The blood picture of pigs kept under conditions favorable to the production and to the prevention of so-called "anemia of suckling pigs." Minn. Agr. Exp. Sta. Tech. Bull. 87.

KNOOP, C. E., W. E. KRAUSS AND R. G. WASHBURN 1935 The development of nutritional anemia in dairy calves. J. Dairy Sci., vol. 18, p. 337.

MCELROY, L. W., AND H. GOSS 1940 A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. II. Vitamin B_6 (pyridoxine). J. Nutrition, vol. 20, p. 541.

MCKIBBIN, J. M., R. J. MADDEN, S. BLACK AND C. A. ELVEHJEM 1939 The importance of vitamin B_6 and factor W in the nutrition of dogs. Am. J. Physiol., vol. 128, p. 102.

NEAL, W. M., AND C. F. AHMANN 1937 The essentiality of cobalt in bovine nutrition. J. Dairy Sci, vol. 20, p. 741.

REID, J. T., C. F. HUFFMAN AND C. W. DUNCAN 1943 A study of poikilocytosis in dairy cattle. J. Dairy Sci., vol. 26, p. 738.

——— 1945 Poikilocytosis in dairy cattle. Arch. Path., vol. 39 (6), p. 351.

SCHEUNERT, A., AND M. SCHIEBLICH 1923 Zur Kenntnis der Vitamine. Über die Bildung von Vitamin B durch obligate Darmbakterien. Biochem. Z., Bd. 139, S. 57.

THEILER, A. H., H. H. GREEN AND P. R. VILJOEN 1915 Contribution to the study of deficiency disease with special references to the lambzietkeproblem in South Africa. Union South Africa Dept. Agr. Research Repts. 3–4, Dir. Vet. Res., p. 3.

VILTER, R. W., H. S. SCHIRO AND T. D. SPIES 1940 Effect of synthetic vitamin B_6 on the hemapoietic system of human beings. Nature, vol. 145, p. 388.

WEGNER, M. I., A. N. BOOTH, C. A. ELVEHJEM AND E. B. HART 1940 Rumen synthesis of the vitamin B complex. Proc. Soc. Exp. Biol. Med., vol. 45, p. 769.

——— 1941 Rumen synthesis of the vitamin B complex on natural rations. Proc. Soc. Exp. Biol. Med., vol. 47, p. 90.

WINTROBE, M. M., R. H. FOLLIS, JR., M. H. MILLER, H. J. STEIN, R. ALCAYAGA, S. HUMPHREYS, A. SUKSTA AND C. E. CARTWRIGHT 1943 Pyridoxine deficiency in swine with particular reference to anemia, epileptiform convulsions and fatty liver. Bull. Johns Hopkins Hosp., vol. 72, p. 1.

FIELD ILLUMINATION AND COMMERCIAL HANDLING AS FACTORS IN DETERMINING THE ASCORBIC ACID CONTENT OF TOMATOES RECEIVED AT THE CANNERY

G. FRED SOMERS, KARL C. HAMNER AND WALTER L. NELSON
Agricultural Research Administration, U.S. Department of Agriculture, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, New York; School of Nutrition,[1] Cornell University, Ithaca

(Received for publication June 22, 1945)

A recent resolution by the American Medical Association's Council on Food establishes for tomato juice a minimum level of 20 mg. of ascorbic acid per 100 ml. to meet the "Accepted" seal of the Association. The establishment of such a standard raises the question as to whether all producers of tomato juice could possibly attain such a level with any degree of consistency. Many studies have indicated the wide variations in the ascorbic acid content of tomatoes and tomato products, but a correlated study of the several factors operating in a specific location has been lacking in most of the reports. It is possible that in certain regions an ascorbic acid level of this magnitude could not be obtained at the present time, even when favorable commercial varieties and good canning management were given primary consideration. For a number of years, the laboratories participating in this investigation have been interested in various aspects of this problem, and the present paper is a report of a correlated study.

Numerous investigators have reported on various factors which influence the ascorbic acid content of tomatoes and tomato products. In a review of the literature, Hamner and Maynard ('42) concluded that fruit development and nutrition of the plant had little effect on the ascorbic acid content of the fruit. Varietal differences of from 13 to 44 mg. per 100 gm. of fruit were reported, and light intensity, season and location, and processing were indicated as factors which markedly influence the ascorbic acid value of the fruit or the product made from the fruit.

Some recent studies by Wade ('42), Renard and Kanapaux ('42), and Kidson ('43) have confirmed the findings that wide differences in as-

[1] This study was supported in part by a grant from the Edward A. Filene Good Will Fund, Inc.

corbic acid content of tomato fruit exist among various varieties. Hamner, Lyon, and Hamner ('42) found that winter-grown fruits had approximately 50% as much ascorbic acid as fruits from plants of the same variety grown in the summer. Holmes, Jones and Ritchie ('43) reported slightly greater differences between winter tomatoes and fully ripe summer tomatoes, than those reported by Hamner et al. The studies of Murphy ('42) and Kidson ('43) have also indicated the large effect that environmental conditions exert on the ascorbic acid content of fruits grown in various localities. Hamner, Bernstein and Maynard

TABLE 1

Ascorbic acid content of commercially canned tomato juice.

SOURCE	NUMBER OF CANS	ASCORBIC ACID Mean ± SD [1]	ASCORBIC ACID Range	SOURCE	NUMBER OF CANS	ASCORBIC ACID Mean ± SD	ASCORBIC ACID Range
Market		*(mg./100 ml.)*	*(mg./100 ml.)*	Cannery [2]		*(mg./100 ml.)*	*(mg./100 ml.)*
Brand 1	5	11.9 ± .54	11.3–12.5	Lot 1	10	16.3 ± .82	15.3–17.8
Brand 2	10	12.1 ± 1.07	10.6–13.9	Lot 2	10	17.7 ± 1.64	15.2–19.3
Brand 3	10	5.7 ± .27	5.0– 6.0	Lot 3	10	16.5 ± .88	15.1–17.5
Brand 4	10	5.7 ± .26	5.4– 6.2	Lot 4	10	17.4 ± .54	16.7–18.4
Brand 5	10	17.8 ± .66	17.0–18.7	Lot 5	10	21.0 ± .09	20.6–21.6
Brand 6	10	6.9 ± .57	6.4– 8.0	Lot 6	10	20.6 ± .88	19.4–21.5
Brand 7	10	7.9 ± 3.92	4.6–13.4	Lot 7	10	19.8 ± 1.52	17.5–21.2
Brand 8	10	14.5 ± .76	13.8–16.1	Lot 8	10	18.5 ± .60	17.7–19.7
				Lot 9	10	19.8 ± .44	19.1–20.4
				Lot 10	10	18.2 ± .28	17.8–18.6
				Lot 11	10	19.3 ± .85	17.8–20.1
				Lot 12	10	18.7 ± .25	18.4–19.0
				Lot 13	10	18.2 ± .85	16.9–19.1
				Lot 14	10	16.2 ± .44	15.6–16.9
				Lot 15	10	19.4 ± 1.26	17.8–22.4
				Lot 16	10	19.1 ± 1.20	16.8–20.4

[1] Standard Deviation $= \sqrt{Sd^2/n-1}$.

[2] The manufacturing process is that of Factory A described by Robinson et al. ('45).

('45) have shown the great influence on the ascorbic acid content of tomatoes produced by variations in light intensity previous to harvest. Such factors as degree of ripeness after the fruit is "mature green," storage of fruit for 10 to 14 days at temperatures from 65° to 90°F., fertilizer treatment, and relative humidity had only a slight effect, if any, on the ascorbic acid content of the fruit.

The work of Bailey ('38) indicated that market tomato juice varies widely in ascorbic acid content, but did not show the extremely low values that have been encountered in other surveys. Recently, Pressley, Ridder, Smith and Caldwell ('44) reported a range of 2.5 mg. to 25.2 mg. per 100 gm. for commercially canned tomato juice. However,

in these studies no data were given relative to the prior treatment of the juice, the exact source of the raw material, or the variation from can to can within a particular brand. Therefore, the factors mainly responsible for the large variations could not be ascertained.

Preliminary investigations carried out in this laboratory on the ascorbic acid content of market tomato juice (table 1) showed that a relatively small can to can variation occurred within a single brand but that wide variations existed among various brands. A survey of the ascorbic acid content of the tomato juice produced at a single cannery [2] also was made. Analyses were made of the juice produced each day the plant was in operation throughout the season. The results, which are presented in table 1, show little can to can variation for any one day, but a somewhat greater variation among days. Even so, the total variation throughout the season was not great as compared to the variation between brands on the retail market. These results indicate that, even with a uniform canning process, there is a considerable variation in the ascorbic acid content of tomato juice. It seems unlikely that this would arise during the processing since the same process was used throughout (see also the accompanying paper by Robinson et al., '45). On the other hand it seems likely that the ascorbic acid content of the tomatoes supplied to the cannery may be a factor. If this is so, variation in the tomatoes may arise from two sources: (1) losses subsequent to picking and before processing, (2) differences in the ascorbic acid content of tomatoes in the field. Some preliminary studies have been made of this aspect of the problem and the following is a report on the findings. At the same time a study was made of the influence of various processing procedures. In this latter study, which was made by Robinson et al. ('45, see accompanying paper), the same source of tomatoes was used, in part, as in the first two experiments in this paper.

Loss of ascorbic acid from the field to the cannery

Experiment 1. On August 31st, the above-mentioned cannery was scheduled to utilize Stokesdale tomatoes from a particular field a short distance away from the cannery. Pickers entered the field in the morning and proceeded to harvest the crop in the usual manner. The following samples were collected between 8:30 and 9:15 A.M. Sample no. 1: Fully ripe, uninjured fruits picked from the vines a short distance

[2] The raw material used by this cannery is discussed in this paper. The type of process used is that of Factory A as described by Robinson et al. ('45) in an accompanying paper. Grateful acknowledgment is made to the assistance and cooperation of Mr. Wilson of the Empire State Pickling Co., Phelps, New York.

ahead of the commercial harvesters. Sample no. 2: Fruit from the boxes into which the commercial pickers were emptying their baskets. In this sample, fruits were selected which showed no injury or which were only very slightly damaged. Sample no. 3: A random sample from these boxes. Damaged as well as uninjured fruit were taken in an effort to get a sample as nearly representative of the harvest as possible. Sample no. 4: Since the portion of the field which was being harvested was infested with weeds, a sample of fruit was taken from an adjacent portion of the same field which had been carefully weeded. All the fruits for this sample were taken from the south side of the vines where the fruits and the adjacent leaves had maximum exposure to sunlight.

The cannery commenced processing fruit from this field about 2 P.M. An additional sample was, therefore, taken at the canning plant as follows. Sample no. 5: A representative sample taken at random from the boxes on the floor of the cannery.

For each sample twenty to thirty fruits were taken, individually wrapped in a large amount of tissue paper and carried to Ithaca for analysis the next day. They were stored overnight at about 40°F. Some of the fruits were soft at the time of analysis. They were all analyzed nevertheless. The fruits of samples 1 and 2 arrived in excellent condition. Evidence from previous work by Hamner et al. ('45), has indicated that the method of harvesting and preparing samples 1 and 2 should not have resulted in any appreciable loss in ascorbic acid.

The method of obtaining the samples of individual fruits and of preparing the acid extract has been previously described (Hamner et al., '42). The samples of the fruit were extracted with a mixture of 2% metaphosphoric acid in N sulfuric acid and ascorbic acid determinations made by a modified xylene method (Bukatsch, '39; Stotz, '41; Nelson and Somers, '45). The results of the ascorbic acid analyses are shown in table 2. There was no significant difference in ascorbic acid content between any of the samples from 1 to 5. Robinson and his co-workers ('45), took samples from the trimming line only a few minutes after our sample no. 5 was taken from the boxes in the canning plant. Their samples were extracted within a few minutes after the sample was taken and were analyzed a few hours later. The ascorbic acid value obtained did not differ significantly from the values obtained in our samples 1 to 5, i.e., they obtained 31 mg. of ascorbic acid per 100 gm. of fresh fruit on this sample. It would appear, therefore, that there was no appreciable loss in ascorbic acid from the field to the trimming line in the cannery in this experiment.

Experiment 2. On September 18th, the cannery was processing the same variety of tomato from another field about half a mile from the above field. A sample of thirty-one fruits collected in the morning from this field gave an ascorbic acid value of 28.5 ± 0.75 mg. of ascorbic acid per 100 gm. A sample of twenty fruits taken at random from the boxes in the canning plant late in the afternoon and extracted immediately for ascorbic acid gave a value of 24.1 ± 0.90 mg., and another sample of twenty fruits taken from the trimming line and extracted in acid at once gave a value of 24.0 ± 1.08 mg.

TABLE 2

Ascorbic acid content (milligrams per 100 gm. fresh fruit) of tomatoes prior to processing.

SAMPLE NO.	DESCRIPTION OF SAMPLE	NO. OF FRUITS	ASCORBIC ACID Mean ± Standard Error
1	Selected ripe fruit from vine	23	30.8 ± 1.26
2	Selected fruit from boxes in field	23	33.2 ± 1.08
3	Random sample from boxes in field	20	32.9 ± 1.17
4	Fruit selected which were exposed to maximum illumination in the weeded portion of the field	21	31.8 ± 0.90
5	Random sample from boxes in canning plant	25	29.0 ± 0.94

In this latter experiment, it appears that there was a significant loss in ascorbic acid between the field and the processing line. It may be recalled that sample no. 5 (table 1) taken from the boxes on the floor of the canning plant was lower in ascorbic acid than the field samples, although not significantly so. It seems possible, therefore, that one might expect some loss in ascorbic acid content of tomatoes during the picking, transportation, and holding of the fruit at the cannery before processing. If the fruits are held overnight or more than a day at the cannery before processing, losses might be considerable, especially if the fruits are injured and in poor condition.

Variations in ascorbic acid content of fresh tomatoes during the canning season and from one field location to another

Since a study was made of variations in ascorbic acid content of canned tomato juice during the course of the canning season at this particular cannery, measurements of the ascorbic acid content of fresh tomatoes from the field were also made during the course of the season.

Thirty ripe fruits, representing a random sampling of the field, were picked on three different dates, one relatively early in the canning season, another at the height of the season, and a third near the end. The ascorbic acid values in milligrams per 100 gm. fresh fruit with the corresponding dates were as follows: August 31st, 30.8 ± 1.26; September 7th, 29.4 ± 0.82; and September 18th, 31.4 ± 1.25. These data are too few to warrant definite conclusions, but they do indicate that there was no general trend in ascorbic acid values during the course of the season, either upward or downward.

The cannery, during the course of the season, obtained fruit from many different fields within a radius of a few miles. Hence it was of interest to see how much variation in ascorbic acid might be found between samples of fruit of the same variety collected from different fields. It was also desirable to determine whether or not such variations as might be found could be correlated with the amount of illumination at each field, since some previous results (Hamner et al., '45) have indicated that the light intensity to which tomato plants are exposed may to a large extent determine the ascorbic acid level in the fruit.

Three fields of tomatoes were selected: one containing Stokesdale tomatoes at Phelps, New York (the field which had been used in connection with the cannery experiments); one with both Stokesdale and John Baer tomatoes at Geneva, New York; and one with John Baer tomatoes at Lincoln, New York.[3]

An integrating light recorder similar to that described by Sprague and Williams ('41) was placed within 0.5 of a mile of each of these fields. These recorders had been calibrated previously at the laboratory, and at the end of the experiment they were again calibrated. It is believed that the comparative values are reasonably accurate. The instrument consists of a photoelectric cell mounted in a spherical globe and an instrument which records a number of counts proportional to the amount of illumination (Sprague and Williams).

The light recorders were in operation for a period of 18 days from September 1st to 18th, inclusive, and daily records were obtained. The following are the comparative values for the entire period: Phelps = 100, Geneva = 67.4, and Lincoln = 71.8. On September 18th, analyses for ascorbic acid content were made on fruits from each of the three fields with the results shown in table 3. The amount of illumination at Geneva was 67.4% of that at Phelps, and the ascorbic acid values for the Stokesdale variety were 75% of those at Phelps. There was almost

[3] Grateful acknowledgment is made to the cooperation of Dr. C. B. Sayre in obtaining these samples.

the same illumination at Lincoln as at Geneva, and the ascorbic acid values were almost identical for the John Baer variety.

The illumination values given above represent average values over the entire experimental period. The daily records showed similar fluctuations at Phelps and Geneva, although the values at Phelps were nearly always slightly higher than those at Geneva on any particular day. The fluctuations from day to day at Lincoln, which is nearly 30 miles northwest of Phelps, followed a somewhat different pattern than at the other two locations. It was surprising that there was so much difference in illumination between Geneva and Phelps which were only 8 miles apart. Geneva is located at the northern end of Seneca Lake, while Phelps is northwest of Geneva. It may be that the proximity of Seneca Lake resulted in the lower illumination values at Geneva.

TABLE 3

Ascorbic acid content and relative illumination of tomatoes grown at three fields.

LOCATION	VARIETY	RELATIVE ILLUMINATION	NO. OF ANALYSES	ASCORBIC ACID CONTENT OF FRESH FRUIT Mean ± Standard Error
				mg./100 gm.
Phelps, N. Y.	Stokesdale	100	27	31.4 ± 1.25
Geneva, N. Y.	Stokesdale	67.4	42	23.6 ± 0.65
	John Baer		42	20.0 ± 0.40
Lincoln, N. Y.	John Baer	71.8	27	19.0 ± 0.59

These data indicate that the variations in ascorbic acid content from one field to another may reflect variations in illumination. The results are in accordance with previous results (Hamner et al., '42; Murphy, '42; Hamner and Maynard, '42) which indicate that the variations in ascorbic acid values from one location to another may be very large and that the intensity of illumination may be the dominant factor in causing the variations (Hamner et al., '45).

DISCUSSION

In considering the losses in nutritive value which might occur during processing of food products, one should bear in mind the possible losses during the picking and handling of the crop prior to its arrival and utilization at the cannery. In comparing the nutritive value of products from various canneries, it is also important to ascertain to what extent the values obtained for a given product are determined by the original values of the fresh material which was used. For example, it seems

possible that the relatively great variations which have been reported for the ascorbic acid values of fresh tomatoes might account for much of the variation which occurs in canned tomato juice.

In this paper, wide variations in ascorbic acid content of canned tomato juice obtained on the market were found. These variations are much greater than can be accounted for on the basis of the experimental variations in the processing methods (see the accompanying paper by Robinson et al., '45). The loss in ascorbic acid during the picking and transportation of the fruit to the cannery was so small in these experiments that it would seem likely that variations in these procedures would not explain the great differences which are found in juice from several sources. The limited storage studies do not provide evidence that variations in storage time or conditions could explain the variable market values.

The work presented here, as well as previous investigations (Hamner et al., '42, '45; Murphy, '42; Hamner and Maynard, '42), indicates that the location or season at which the fruits are produced causes very great variations in the ascorbic acid content of fresh tomatoes. These variations are of sufficient magnitude to provide a possible explanation of the variations in market tomato juice. Differences between varieties doubtless provide for part of the variations.

SUMMARY

1. Little loss in ascorbic acid in tomatoes was found during the picking and transporation of the fruit to the cannery.

2. Considerable variation in ascorbic acid content was found from one tomato field to another.

3. The data presented indicate that the amount of light for 18 days prior to harvest is closely correlated with the ascorbic acid content of tomatoes under field conditions.

4. Differences in amount of illumination appear to account for much of the variation in ascorbic acid content of tomatoes which is found between fields several miles apart.

5. It is suggested that the variations in the fresh tomatoes may be an important factor in determining the variations observed in the ascorbic acid content of market tomato juice.

LITERATURE CITED

BAILEY, E. M. 1938 Tomato juice, canned. Conn. Agr. Exp. Sta. Bull. no. 415, p. 698.

BUKATSCH, F. 1939 Eine neue Methode des C-Vitamins mit dem Stufenphotometer von Zeiss. Z. Physiol. Chem., Bd. 262, S. 20.

HAMNER, K. C., LEON BERNSTEIN AND L. A. MAYNARD 1945 Effects of light intensity, day length, temperature and other environmental factors on the ascorbic acid content of tomatoes. J. Nutrition, vol. 29, p. 85.

HAMNER, K. C., C. B. LYON AND C. L. HAMNER 1942 Effect of mineral nutrition on the ascorbic acid content of the tomato. Bot. Gaz., vol. 103, p. 586.

HAMNER, K. C., AND L. A. MAYNARD 1942 Factors influencing the nutritive value of the tomato. U.S.D.A. Misc. Pub., no. 502.

HOLMES, A. D., C. P. JONES AND WALTER S. RITCHIE 1943 The ascorbic acid content of late winter tomatoes. New Eng. J. Med., vol. 229, p. 461.

KIDSON, ELSA B. 1943 Vitamin C content of different tomato varieties grown in the Nelson District. New Zealand J. Sci. Tech., 25 B, p. 129.

MURPHY, ELIZABETH F. 1942 The ascorbic acid content of different varieties of Maine grown tomatoes and cabbages as influenced by locality, season, and stage of maturity. J. Agr. Res., vol. 64, p. 483.

NELSON, WALTER L., AND G. FRED SOMERS 1945 The application of the xylene method to the determination of ascorbic acid in large numbers of tomato and tomato juice samples. Ind. Eng. Chem., Anal. Ed. In press.

PRESSLEY, ANNE, CLARA RIDDER, M. C. SMITH AND EMILY CALDWELL 1944 The nutritive value of canned foods. II. Ascorbic acid and carotene or vitamin A content. J. Nutrition, vol. 28, p. 107.

REYNARD, G. B., AND MARGARET S. KANAPAUX 1942 Ascorbic acid (vitamin C) content of some tomato varieties and species. Proc. Am. Soc. Hort. Sci., vol. 41, p. 298.

ROBINSON, W. B., ELMER STOTZ AND Z. I. KERTESZ 1945 The effect of manufacturing methods on the ascorbic acid content and consistency characteristics of tomato juice. J. Nutrition, vol. 30, p. 435.

SPRAGUE, V. G., AND E. M. WILLIAMS 1941 An inexpensive integrating light recorder. Plant Physiol., vol. 16, p. 629.

STOTZ, ELMER 1941 A clinical method for the determination of ascorbic acid in blood plasma and urine. J. Lab. and Clin. Med., vol. 26, p. 1542.

WADE, B. L. 1942 The relationship of variety and environment to the nutrient content of vegetables grown in the South. Assn. Southern Agr. Workers, Proc. Ann. Convention, vol. 43, p. 168.

THE EFFECT OF MANUFACTURING METHODS ON THE ASCORBIC ACID CONTENT AND CONSISTENCY CHARACTERISTICS OF TOMATO JUICE [1, 2]

WILLARD B. ROBINSON, ELMER STOTZ AND Z. I. KERTESZ

New York State Agricultural Experiment Station, Cornell University, Geneva, New York

(Received for publication June 22, 1945)

The accompanying paper by Somers, Hamner and Nelson ('45) illustrates the great variations which may be found in the ascorbic acid content of market samples of tomato juice, and the relation of these variations to growing conditions, harvesting, and delivery of tomatoes to the processing plant.

The ascorbic acid level of tomato juice purchased on the market will be governed by three main factors, namely (1) the ascorbic acid content of the raw product, (2) the effect of production methods, and (3) the influence of storage on the finished product. This paper presents the results of studies on ascorbic acid losses during processing in two commercial canning plants, with special reference to the preheating temperatures employed during crushing of the tomatoes and to related changes in the consistency of the juice produced. In this paper the term "breaking" refers to the crushing of tomatoes prior to extraction of the juice, "hot break" when heat is simultaneously applied during the crushing, "cold break" when no added heat is employed. Storage studies of the tomato juice produced under controlled conditions are in progress and will be presented in another report.

There are several older references to the ascorbic acid losses which may occur in the preparation of tomato juice. Some of these investigators employed insufficiently accurate bioassay methods for determination of the vitamin and most of the studies were not carried out under commercial production conditions. The general conclusion is prevalent, however, that the processing loss is small when conditions avoiding oxidation are observed (Kohman, Eddy and Gurin, '33; Daggs and Eaton, '34). Sanborn ('38) has given particular emphasis to the

[1] Journal Paper no. 632, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

[2] This work was supported in part by a grant from the Cooperative G.L.F. Exchange, Inc., Ithaca, New York.

increased losses which may occur when the hot juice is exposed excessively to aeration, for example during centrifugal pumping and tank holding of the juice. One of the few actual figures reported in this survey cites a 19% loss of ascorbic acid during a 13-minute period required for the heating of juice in a tank prior to can filling. Sanborn stated that the most complete conservation of vitamin C was found in plants employing the "hot break" of the tomatoes.

Kertesz and Loconti ('44) have studied the factors which govern the consistency of tomato juice, and demonstrated that application of the "hot break" method gives a juice of superior consistency. The wide use of tomato juice as an important nutritional source of vitamin C is contingent on maintaining satisfactory color, consistency, and flavor in the juice. Since considerations of juice consistency advise the "hot breaking" of tomatoes, it was important to determine whether the elevated temperature (up to 200°F.) employed would cause more loss of ascorbic acid than if the juice were prepared without heat or with only preheating. Opposed to the possibility of increased destruction due to elevated temperatures, the presence in the tomato skin of thermo-labile agents which facilitate the destruction of ascorbic acid (Wokes and Organ, '43), could be suspected of causing more rapid loss of ascorbic acid during the manufacture by a "cold break" method. Particular attention was therefore given in this study to the effect of preheating temperatures on the ascorbic acid content of the processed tomato juice. Simultaneous observations on the consistency of these juices were made to determine whether any relation exists between consistency and the stability of the ascorbic acid.

The experiments were carried out in two commercial canning plants during regular production in the 1944 season.[3] One of these employed the "hot break" procedure and here it was possible to study the effect of different preheating temperatures without other changes in the procedure. In the second plant where the "cold break" process was used, conditions favored a study of the effect of tank holding and processing times on the ascorbic acid content of the finished juice.

EXPERIMENTAL PROCEDURES

The manufacturing procedure used in factory A was as follows: The tomatoes were washed, sorted, and trimmed in the usual manner. From the sorting table the fruit entered a crusher-preheater.[4] This

[3] We are grateful to Mr. Floyd Wilson of the Empire State Pickling Co., Phelps, New York, and to Mr. Irving B. Cook of the G.L.F. plant in Waterloo, New York, for their assistance and cooperation.

[4] Food Machinery Corp., 40-gallon per minute capacity.

was of tubular construction and contained a worm revolving at 184 RPM which crushed the tomatoes and forced them through the tube. With a given rate of steam supply to the preheater, the extent of heating depended somewhat on the volume of tomatoes. Preheating temperatures were varied by regulating the steam supply to the breaker, and recorded by some 40 to 60 temperature readings on the material emerging from the breaker. The crushed tomatoes then entered a juice extractor [5] in which the juice was pressed through a closely fitting screen by a screw revolving at 300 RPM. The juice was pumped into a 200-gallon stainless steel tank, heated to 180°F., and filled into no. 2 size cans. These were sealed on a high speed closing machine, processed for 5 minutes in a tank of hot water (190°F.), and finally cooled to about 130°F.

In factory B the tomatoes were trimmed, crushed, and the juice extracted entirely without the application of heat. The juice was pumped into 600-gallon stainless steel tanks and then heated to 180°F. with continuous stirring. Usually the juice was held for at least 15 minutes before filling into no. 3 (tall) size cans. These were processed for 20 minutes in boiling water and cooled for several hours in a cold water tank.

Temporary interruptions in the production line made it possible to relate all samples taken along the processing line to the particular lot of fruit used as raw product. In practice, the preheater, juicer, and a receiving tank were emptied before starting a given temperature run. A batch of tomatoes, all collected from the same field was then prepared and fed to the preheater, and approximately thirty whole fruits removed from the trimming line at random (series A, table 1) during the 20 to 25 minutes required for withdrawing samples at other points in the processing line. Comparable lots of the raw product for each temperature run was thereby afforded.

The second series, consisting of at least four samples, was taken as the crushed fruit emerged from the crusher-preheater (series B, table 1). These samples were preserved in metaphosphoric acid immediately to serve for subsequent determination of ascorbic acid.

The third series of samples was taken at the filling machine in factory A, and from the tank after heating to 180°F. in factory B (series C, table 1) and consisted of two portions in each case. A fourth series of samples was taken from two cans after the cooling operation (series D, table 1).

[5] Food Machinery Corp., "Super Pulper," 20 tons per hour.

The trimmed tomatoes were sampled and preserved for ascorbic acid analysis at the factory by cutting a slice of approximately 25 gm. from the center of the tomato, and homogenizing with 100 ml. of 2% metaphosphoric acid in 1 N sulfuric acid in a Waring Blendor. Other samples from the processing lines were preserved immediately by pipetting 5 ml. of the juice into 10 ml. of the acid mixture. The preserved samples were analyzed for ascorbic acid either immediately on return to the laboratory or within 24 hours after storage in a cold room. Preliminary investigations had shown that the latter procedure involved no loss of ascorbic acid. Ascorbic acid was determined in duplicate on each filtered extract by the xylene extraction method of Robinson and Stotz ('45). The formaldehyde modification of this method showed that all of the indophenol reducing power of the filtrate was due to ascorbic acid.

The determination of various factors related to the consistency of the juice was made by methods described previously (Kertesz and Loconti, '44). Viscosity of the juice ("gross viscosity") was determined in the Stormer viscosimeter at 30°C. using a 90 ml. sample and 50 gm. driving weight. The serum viscosity was determined at the same temperature in an Ostwald viscosimeter. The calcium pectate content of the serum was determined by the Poore ('34) modification of the Carre and Haynes method ('22), using a preliminary precipitation with 70% ethanol containing 0.05 N hydrochloric acid. The rate of filtration was determined by observing the proportion of 100 ml. of the juice which passed through a 18.5 cm. Whatman no. 12 filter paper in 15 minutes.

RESULTS

Table 1 summarizes the ascorbic acid results and physical characteristics of the juices prepared in factories A and B at the different preheating temperatures. The relation between preheating temperatures and the consistency of the juice was in agreement with previous observations (Kertesz and Loconti, '44). Average figures are recorded for ascorbic acid contents with probable errors for the analyses of whole tomatoes, and ranges for the juice samples. Runs I, II, and III were carried out in plant A on the same day. Variations from 156°–200°F. in the preheating temperatures caused no significant differences in the ascorbic acid content of samples taken at any point in the processing line. A 15 to 19% loss in ascorbic acid was observed in the overall process, nearly all of this occurring during the tomato crushing stage. On another day at plant A, the breaking temperature was lowered to

include juice prepared at 80°F., without added heat. Again there was no relation of the preheating temperature to the ascorbic acid content of the finished tomato juice. In run VII, carried out in plant B, only the "cold break" procedure was used. In spite of differences in the type of breaking and juicing, longer standing in the tanks, and a longer processing time, there was nevertheless approximately the same loss of ascorbic acid during the overall manufacturing procedure. In this plant the greatest loss occurred after the juicing procedure rather than during the breaking operation.

TABLE 1

The relation of preheating temperatures to ascorbic acid losses in the processing line and to consistency characteristics of the juice.

RUN [1]	PREHEATING TEMP.	AVERAGE ASCORBIC ACID (MG./100 GM.) [2]				PROCESSING LOSS	RELATIVE VISCOSITY [3]		CALCIUM + [4] PECTATE IN SERUM	COMPARATIVE FILTRATION RATE
		Series A	Series B	Series C	Series D		Juice (Stormer)	Serum (Ostwald)		
	°F.					%			%	
I	200 (185–203)	31.3 ± 0.65 [5]	27.4	26.0	26.4	15.6	2.20	1.82	.100	6.0
II	170 (168–184)	31.8 ± 0.62	27.3	24.2	25.8	18.8	1.76	1.57	.087	8.0
III	156 (148–176)	31.3 ± 0.69	24.8	26.5	26.8	14.5	1.58	1.32	.038	11.0
IV	185 (170–206)	33.5 ± 0.56	28.2	27.0	27.0	19.4	2.02	1.94	.089	7.0
V	124 (98–146)	Same as IV	28.1	27.6	27.3	18.5	1.58	1.44	000	11.7
VI	80 (76–84)	Same as IV	28.0	27.6	27.5	17.9	1.59	1.45	.000	12.7
VII	80	20.8 ± 0.46	20.7	16.8	16.6	20.2	1.26	1.15	.000	33.0

[1] Runs I–III on same day at plant A, IV–VI on a different day; VII at plant B.

[2] Series A — trimmed tomatoes; series B — after breaking; series C — before can filling; series D — processed juice.

[3] For particulars of the methods see the text.

[4] After preliminary precipitation with acid ethanol.

[5] Probable error of the mean.

The ascorbic acid content of the raw tomatoes was considerably less at plant B than at plant A, resulting in juice with a considerably lower ascorbic acid content. The accompanying paper of Somers, Hamner and Nelson ('45) offers a possible explanation of this finding involving differences in solar illumination prior to harvesting.

In contrast to the negative effect of breaking temperature on the ascorbic acid content of the juice, a distinct effect is noted on consistency characteristics of the juice. Juices prepared by the "cold break" method possessed a low viscosity and high filtration rate. Juice prepared in run VII showed a separation of a watery layer from the juice on standing. No pectic substances could be found in the serum of the juices prepared with preheating temperatures of 124°F. or lower, results which confirm more controlled laboratory experiments (Kertesz and Loconti, '44).

TABLE 2

Effect of heating in an open tank and of hot water processing of canned tomato juice on the ascorbic acid content.

TREATMENT	TIME	ASCORBIC ACID	LOSS IN PROCESSING
	min.	*mg./100 ml.*	%
Juice standing in open tank at 180°F. with constant stirring — Duplicate analyses.	0	15.5	..
	4	15.0	3.2
	10	14.6	5.8
	30	15.0	3.2
	60	13.1	15.5
	90	12.8	17.4
	120	11.1	28.4
Cans processed in boiling water tank. Duplicate cans analyzed 4 days after processing.	0	13.5	..
	10	13.4	0.7
	20	13.3	1.5
	30	13.5	0.0
	60	12.9	4.4
	90	12.4	8.1
	120	12.2	9.6

Since no outstanding differences in the ascorbic acid content of the finished juice could be attributed to variations in the preheating temperatures, the effect of exposure of the juice to air in the heating tank and prolonged processing in the closed can was studied. These experiments were carried out in plant B, where the tomato juice was kept in open 600-gallon stainless steel tanks at 180°F. and continuously stirred. The canned juice (no. 3 size cans) was processed in a tank of boiling water. The results of these experiments are shown in table 2. It is evident that the hot juice exposed to air lost considerable ascorbic acid, amounting to approximately 30% in 2 hours. Excessively long storage of the hot juice in a tank could therefore lead to production of a juice with a considerably lower ascorbic acid content. There was little loss during processing in the sealed can. During the 20 minutes of process-

ing regularly employed at plant B, there was no loss of ascorbic acid; 1 hour of heating resulted in 4% loss, and 2 hours in approximately a 10% loss.

DISCUSSION

The experiments reported show that under efficient manufacturing conditions, tomato juice should contain at least 80% of the ascorbic acid content of the trimmed tomatoes. Wide variations in the preheating temperature, although causing great differences in the consistency, produced no changes in the losses of ascorbic acid during the manufacture of tomato juice. In general, the great stability of ascorbic acid in tomato juice in contrast to its stability in other types of solution is noteworthy. There are apparently no enzyme systems in the juice which are effective in the oxidation of vitamin C; in fact there appears to be present an efficient inhibitor of ascorbic acid oxidation.

Assuming efficient manufacturing conditions, it appears that the greatest single factor in producing juices of widely different vitamin C contents is the amount of vitamin in the tomatoes. The wide variation in market samples of tomato juice (Pressley et al., '44) as contrasted to the smaller variation in tomato juice samples collected at the cannery (Somers, Hamner and Nelson, '45), might be traced to differences in storage conditions as well as to variations in the fruit.

Recently the Council on Foods and Nutrition of the American Medical Association ('45) proposed to place the "seal of acceptance" on canned tomato juice only if this contained in excess of 20 mg. of ascorbic acid per 100 ml. juice. It is clear that in order to obtain freshly made tomato juice which will meet this standard, the tomatoes used in the manufacture must contain an average of at least 25 mg. of ascorbic acid per 100 gm. (trimmed) fruit. In view of inevitable losses of ascorbic acid during storage and distribution, it may also be necessary to specify favorable storage conditions before a "seal of acceptance" can become an effective nutritional measure.

SUMMARY

1. Losses of ascorbic acid were measured during the commercial production of tomato juice, with particular attention to possible variations due to different preheating temperatures.

2. An overall ascorbic acid loss of 15 to 19% occurred during tomato juice manufacture, and this was independent of the preheating temperature. A distinct effect of preheating temperature on consistency characteristics of the juice was evident.

3. The principal loss of ascorbic acid occurred during the "breaking" of the tomatoes. Considerable loss may occur during excessive holding of the hot juice in an open tank, but little during processing in the can.

LITERATURE CITED

CARRE, M. H., AND D. HAYNES 1922 The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble pectin in apples. Biochem. J., vol. 16, p. 60.

COUNCIL ON FOODS AND NUTRITION 1945 Acceptability of foods with high natural vitamin C content. J. Am. Med. Assn., vol. 128, p. 204.

DAGGS, R. G., AND A. G. EATON 1934 Vitamin C content of canned tomato juice. Ind. and Eng. Chem., vol. 26, p. 292.

KERTESZ, Z. I., AND J. D. LOCONTI 1944 Factors determining the consistency of commercial canned tomato juice. New York State Agr. Exp. Sta. Tech. Bull. no. 272.

KOHMAN, E. F., W. H. EDDY AND C. Z. GURIN 1933 Vitamins in canned foods. XIII. Canning tomato juice without vitamin C loss. Ind. and Eng. Chem., vol. 25, p. 682.

POORE, H. D. 1934 Recovery of naringen and pectin from grapefruit residue. Ind. and Eng. Chem., vol. 26, p. 637.

PRESSLEY, A., C. RIDER, M. C. SMITH AND E. CALDWELL 1944 Nutritive value of canned foods. II. Ascorbic acid and carotene or vitamin A content. J. Nutrition, vol. 28, p. 107.

ROBINSON, W. B., AND E. STOTZ 1945 The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances. J. Biol. Chem., vol. 160, p. 217.

SANBORN, N. H. 1938 Conservation of vitamin C in tomato juice production. The Glass Packer, vol. 17, p. 102.

SOMERS, G. F., K. C. HAMNER AND W. L. NELSON 1945 Field illumination and commercial handling as factors in determining the ascorbic acid content of tomatoes received at the cannery. J. Nutrition, vol. 30, p. 425.

WOKES, F., AND J. G. ORGAN 1943 Oxidizing enzymes and vitamin C in tomatoes. Biochem. J., vol. 37, p. 259.

THE ELIMINATION AND DISTRIBUTION OF SELENIUM IN THE TISSUES IN EXPERIMENTAL SELENIUM POISONING [1]

IRENE ROSENFELD AND O. A. BEATH
Department of Research Chemistry, University of Wyoming, Laramie

(Received for publication June 12, 1945)

The retention and elimination of selenium from the tissues of livestock raised on seleniferous areas are important from the public health point of view. Smith et al. ('36) found that the rural population of eastern Wyoming, South Dakota, and northern Nebraska excreted from 100 to 200 µg. % of selenium. Normally, human urine in nonseleniferous areas contains no selenium. Smith and his co-workers ('37) reported that the probable source of selenium was of animal origin, such as milk, eggs, and meat as well as cereal grains and vegetables. They reported that the incidence of vague symptoms of ill health, suggestive of gastric and hepatic disorders, were high enough to indicate the probability of cause and effect.

Investigations dealing with the elimination and distribution of selenium on laboratory animals were carried out by Smith et al. ('37, '38) and Anderson and Moxon ('41). Studies dealing with the distribution and elimination of selenium in farm animals are few. Dudley ('36) reported the distribution of selenium in cases of acute and subacute selenium poisoning in livestock. However, he did not study the elimination of selenium in these animals. Since seleniferous areas supply a large amount of our meat, it appeared to us that an investigation on the elimination and distribution of selenium in the tissues of larger animals should merit consideration. Our experiments were designed to study the effect of protein diets upon selenium toxicity, the biochemical changes which occur in selenium poisoning and the distribution and elimination of selenium. The results of the first-mentioned problem were reported elsewhere (Rosenfeld and Beath, '46). In this paper we wish to report on the elimination and distribution of selenium in the different organs.

[1] Approved for publication by the Director of the Wyoming Experiment Station.

EXPERIMENTAL

Yearling ewes raised in Wyoming were used as experimental animals. The ewes were divided into four groups, five animals in each group. Group I received a high protein diet (20% digestible protein); group II was fed with a medium protein diet (12% digestible protein); group III was given a low protein diet (1.5% digestible protein); and group IV, controls, received the same diets without selenium. The percentage composition of the respective diets is given in table 1.

TABLE 1

Percentage composition of diets fed to sheep.

INGREDIENT	HIGH PROTEIN	MEDIUM PROTEIN	LOW PROTEIN
	%	%	%
Ground yellow	30	12.5	
Linseed meal	25	16	
Cottonseed meal	18	10	
Wheat bran	13	12.5	
Molasses	10	20	19
Mineral mixture[1]	4	4	4
Wheat straw		25	25
Dried starch waste			25
Cottonseed hull			25
Corn oil			2

[1] Ground limestone 40%, disodium phosphate 40%, and iodized salt 20%.

The selenium was given by drenching. Water extract of the seleniferous plant, Atriplex canescens, was used as a source of organic selenium. The concentration of selenium in the extract was determined by the method of Klein ('41). However, we found that whether we used organic or the inorganic selenium of sodium selenite (Na_2SeO_3) acute intoxication occurred with the same dose when the selenium was administered orally. Therefore, at the end of the experiment we used the inorganic selenium. Each experimental animal was drenched with 10 mg. of selenium, daily for 21 days, and the dose was increased to 20 mg. daily until the animals developed toxic symptoms. In two intoxicated animals of each group the selenium feeding was discontinued, and in three sheep the dose was increased to 30 mg. daily. The selenium administration was stopped in order to study its elimination from the different tissues and to determine the length of time required for the animals to be free of urinary selenium. Once a week each sheep was placed in a metabolism cage for 24 hours and urine collected with toluol as the preservative. The total quantity of urine excreted was noted and the amount of selenium was calculated on that basis. Blood was taken

from the jugular vein of the animals. Analyses of selenium in the blood, urine and tissues were carried out according to the method of Klein ('41).

DISCUSSION

Urinary excretion of selenium of the three groups of animals which received selenium until death are given in table 2. Groups on high, medium and low protein diets excreted 4.8, 5.5 and 3.5 mg. of selenium, respectively, during 33 days. However, as the administration of selenium was continued, there was a decreased elimination, with subsequent oliguria, and anuria. This decrease was associated with kidney injury as reported by Draize and Beath ('35).

TABLE 2

Excretion of selenium in the urine by the different diet groups. Sheep received selenium until death.

HIGH PROTEIN DIET Selenium Administered		Excreted		MEDIUM PROTIEN DIET Selenium Administered		Excreted		LOW PROTEIN DIET Selenium Administered		Excreted	
No. days	*mg./day*	*mg./day*	*%*	*No. days*	*mg./day*	*mg./day*	*%*	*No. days*	*mg./day*	*mg./day*	*%*
0	0 (3)[1]		0	0	0 (3)[1]		0	0	0 (3)[1]		0
33	15.0	4.8	31.7	35	15.0	5.5	36.9	29	15.0	3.5	23.1
51	20.0	5.7	28.7	50	20.0	5.7	28.5	40	20.0	.1.9	9.6
67	30.0	5.7	19.1	64	30.0	5.3	17.5	48	20.0	2.1	10.5
80	30.0	4.7	15.5	78	30.0	4.7	15.5				
107	30.0	4.7	15.6	108	30.0	2.8	8.3				

[1] Numbers in parentheses indicate the number of animals used.

Table 3 gives the selenium in the urine after selenium feeding was discontinued. Before symptoms of intoxication appeared the high and medium protein diet groups had received in gradual doses a total of 860 mg. and the low protein diet group a total of 360 mg. of selenium. The groups which received the higher quantity of selenium eliminated larger amounts. However, 36 to 38 days after the cessation of selenium administration, all groups eliminated approximately the same amount, and at the end of 50 days all the urines were free or contained only traces of selenium. Smith and his co-workers ('37) reported that cats eliminated the bulk of the selenium within 2 weeks. McConnell ('41), using radioselenium as tagged atoms, studied the distribution and elimination of a single subtoxic dose of selenium in rats and found that from 41 to 43% of the original dose appeared in the urine during the first 24-hour period. We have found that sheep excreted only small amounts of selenium in the first 24 hours. It seems that among different species of

animals there is much variation in the time taken before selenium appears in the urine and time required for its depletion. Therefore, the elimination of selenium in smaller animals can serve only as an approximate guide in the larger animals.

TABLE 3

Elimination of selenium in the urine after selenium feeding discontinued.

	ANIMALS RECEIVING DIETS								
	HIGH PROTEIN			MEDIUM PROTEIN			LOW PROTEIN		
		Selenium			Selenium			Selenium	
	Days	Total amount given	Excreted	Days	Total amount given	Excreted	Days	Total amount given	Excreted
		mg.	*mg./day*		*mg.*	*mg./day*		*mg.*	*mg./day*
Duration of selenium feeding	44	860(2)[1]	5.558	44	860(2)[1]	5.98	44	360(2)[1]	2.85
Selenium was discontinued	8		1.20	8		1.20	13		0.21
Selenium was discontinued	36		0.243	36		0.34	38		0.28
Selenium was distinued	50		Traces	50		Traces	52		0.0
Control		0	0.0		0	0.0		0	0.0

[1] Numbers in parentheses indicate the number of animals used.

Table 4 presents the concentration of selenium in the tissues of animals which received selenium until death and the selenium in the tissues of animals in which the selenium feeding was discontinued for 61 and 64 days. Sheep fed with high and medium protein diets were able to withstand a larger amount of selenium than those on the low protein ration. The increased selenium intake in the higher protein diet groups produced a correspondingly increased storage in the different organs. In all groups the liver and kidney contained the largest amount and the muscle and brain stored the smallest amount of selenium. It is interesting to note that the fat was free of selenium in all groups, regardless of the amount of selenium given. This suggests that a greater deposition of selenium occurred in the organs which contained the higher percentages of protein. Dudley ('36) suggested a replacement of sulfur by selenium in certain amino acids which were utilized in the formation of modified proteins. We found that the livers of animals which died

TABLE 4

Selenium in body tissues. (Parts per million.)

DIET	TOTAL SELENIUM GIVEN		LIVER	KIDNEY	HEART	LUNG	SPLEEN	BRAIN	STOMACH	MUSCLE	FAT	BLOOD
	mg.	*days*										
High protein diet												
1. Se given until death	1922 (3)[1]	116	29.21	3.95	1.75	3.10	3.51	0.8	3.9	0.8	0	3.1
2. Se given until intoxicated then discontinued for 61 days and animal killed	860 (2)	44	1.33	1.47	.99	.48	.69	0	.32	0	0	.42
Medium protein diet												
1. Se given until death	1922 (3)	116	33.17	13.55	9.92	4.91	9.54	. .	5.9	0.97	0	1.34
2. Se given until intoxicated then discontinued for 61 days and animal killed	860 (2)	44	1.56	1.06	.26	.67	.71	0	.45	0	0	.61
Low protein diet												
1. Se given until death	632 (3)	64	9.10	6.87	1.90	2.67	1.96	.	.	0.8	. .	2.14
2. Se given until intoxicated then discontinued for 64 days and animal killed	360 (2)	44	1.85	1.57	.57	.38	.67	. .	.	0	.	.74
Control	0		.75	0.5	0.0	0.2	0.3	. .	. .	0		.59

[1]Numbers in parentheses indicate the number of animals used.

of selenium poisoning showed a decrease in the protein and sulfur content regardless of diet (Rosenfeld and Beath, '46), indicating a possible protein-sulfur-selenium antagonism. In sheep in which the selenium feeding was discontinued for 61 and 64 days the selenium content of the tissues was approximately the same as in the control animals, with the exception of the liver and the kidney. The residual selenium present in the different tissues after discontinuance of selenium feeding was considered negligible since such small quantities of selenium would be too low to produce harmful effects. Smith and his co-workers ('40) reported that an estimated intake of about 1.0 to 1.5 mg. per kilogram per day of inorganic selenium was required to produce important pathologic changes in experimental animals. Our results agree with their observations. Also it can be seen from table 4 that if animals were slaughtered at a time when their selenium intake was high, then the meat obtained can be a source of a large amount of selenium in the human diet. The absence of selenium in the urine can serve as a guide for the selenium depletion in the different organs.

SUMMARY

The elimination and distribution of selenium in sheep receiving graded doses of selenium for 44 and 116 days was studied. Sheep fed with selenium until death eliminated smaller quantities of selenium in the urine toward the end, due to kidney injury, and stored larger amounts in all the tissues.

Selenium was excreted in the urine gradually after selenium administration was discontinued. In 50 days the urine showed only traces or was free of selenium.

The storage of selenium in the different organs depended upon the amount of selenium administered to the animals. In all groups the liver and the kidney contained the largest amount and the brain and muscle the smallest amount of selenium. Other organs showed variations as to the quantity of selenium accumulated. The fat was free of selenium regardless of the selenium intake.

A small amount of selenium was present in all the tissues 61 and 64 days after selenium administration was discontinued. According to our present knowledge this amount was considered insufficient to cause harmful effects.

LITERATURE CITED

ANDERSON, H. D., AND A. L. MOXON 1941 The excretion of selenium by rats on a seleniferous wheat ration. J. Nutrition, vol. 22, p. 103.

DRAIZE, J. H., AND O. A. BEATH 1935 Observations on the pathology of blind staggers and alkali disease. J. Am. Vet. Med. Assn., vol. 86, p. 753.

DUDLEY, H. C. 1936 Toxicology of selenium. I. A study of the distribution of selenium in acute and chronic cases of selenium poisoning. Am. J. Hygiene, vol. 23, p. 169.

KLEIN, A. K. 1941 Report on selenium. J. Am. Off. Agr. Chem., vol. 24, p. 363.

McCONNELL, K. P. 1941 Distribution and excretion studies in the rat after a single subtoxic subcutaneous injection of sodium selenate containing radioselenium. J. Biol. Chem., vol. 141, p. 427.

ROSENFELD, I., AND O. A. BEATH 1946. I. The influence of protein diets on selenium poisoning. II. Influence of protein diets on selenium poisoning. The chemical changes in the tissues following selenium administration. Am. J. Vet. Research, vol. 7, (in press).

SMITH, M. I., K. W. FRANKE AND B. B. WESTFALL 1936 The selenium problem in relation to public health. U. S. Pub. Health Rep., vol. 51, p. 1496.

SMITH, M. I., E. F. STOHLMAN AND R. D. LILLIE 1940 Studies in chronic selenosis. National Institute of Health, Federal Security Agency, Public Health Service, part 1, Bull. 174.

SMITH, M. I., AND B. B. WESTFALL 1937 Further studies on the selenium problem in relation to public health. U. S. Pub. Health Rep., vol. 52, p. 1375.

SMITH, M. I., B. B. WESTFALL AND E. F. STOHLMAN 1937 The elimination of selenium and its distribution in the tissues. U. S. Pub. Health Rep., vol. 52, p. 1171.

——— 1938 Studies on the fate of selenium in the organism. U. S. Pub. Health Rep., vol. 53, p. 1199.

SECRETION OF VITAMIN D IN MILKS OF WOMEN FED FISH LIVER OIL [1] [2]

LOUIS J. POLSKIN, BENJAMIN KRAMER AND ALBERT E. SOBEL
Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, Brooklyn, New York

(Received for publication April 30, 1945)

The purpose of the experiments reported in this paper was to determine the vitamin D content of human milk following the ingestion of fish oils containing the antirachitic vitamin.

The literature revealed scanty information on the vitamin D content of human milk and the data were difficult to interpret. Of the normal values given in thirteen papers eleven are reported as either no vitamin D or traces only. Van Niekerk ('33) reported 15 to 22 international units (I.U.) per liter. Drummond, Gray and Richardson ('39) reported an average result of 60 I.U. per liter, the range of the values being from 20 to 90. The results in the fortification experiments reported (eighteen papers) are qualitative rather than quantitative and indicate failure to increase the vitamin D content following the administration of fish oils. We therefore planned an experiment in which massive doses were to be supplied.

PLAN OF EXPERIMENT

The mothers were chosen at random from our prenatal clinic; the study was made during the winter of 1941–1942. The diet prescribed was high in protein, low in fat. The carbohydrate recommended was high initially and was gradually decreased. A liberal supply of milk, green vegetables and eggs was part of this diet. The degree of adherence of the mothers to this prenatal diet could not be checked.

Vitamin D in the form of mixed fish liver oil,[3] bioassayed by us, was supplied in a single dose of 32,000 U.S.P. XI units by capsule at each weekly visit to the clinic. Many mothers failed to attend regularly.

[1] These investigations were aided by a grant from Mead Johnson and Company, Evansville, Indiana.

[2] Presented before the meeting of the American Society of Biological Chemists, held in absentia, 1943. Polskin, L. J., B. Kramer and A. E. Sobel, 1943, Secretion of vitamin D in milks of humans fed fish oil. Fed. Proc., vol. 2, p. 68.

[3] The fish liver oil was kindly contributed by Mead Johnson and Company.

During the observation periods, from 32,000 to as much as 480,000 units were consumed. A total of fifty-nine patients were studied in all, twenty-one of whom served as controls who received no vitamin D supplement. Daily milk collections of from ½ to 2½ ounces were made by means of an electrically-driven breast pump during the first 7 to 9 days of lactation. In one case (L. G., no. 55) an 8-day balance study of the amount of the vitamin present in the blood, milk and stools was made.

Bioassay of vitamin D

The rat bioassay method (A.O.A.C., '40) was used to determine the vitamin D content of the milk, eight feedings being made during the first 8 days; the animals were sacrificed at the end of the tenth day. In order to improve the well-being of the test animals, Steenbock's diet no. 2965 was modified by incorporating 3% brewer's yeast[4] at the expense of the yellow corn in the diet. Sobel, Goldfarb and Kramer ('35), Natelson and Sobel ('35) and Bechtel and Hoppert ('36) reported the incorporation of yeast powder for similar reasons. The bones were rated independently by two of us, following essentially the gradations described by Russell, Wilcox, Waddell and Wilson ('34), and then prepared for photographing by the procedure of Taylor, Klein and Russell ('38).

Vitamin D was determined in blood as follows: For the preliminary blood sample 6 ml. of whole blood were mixed with about 35 gm. of the modified no. 2965 diet. This was entirely consumed by the test rat by the seventh day. The basal ration was then supplied until the animals were sacrificed. For bloods obtained following vitamin D supplementation, high vitamin values were anticipated and, therefore, the whole blood was immediately frozen and dehydrated by the vacuum drying method of Cooper and Grabill ('39) as modified by Warkany, Guest and Grabill ('42). Following preliminary test levels, 350 mg. of blood solids were mixed into the 2965 ration as described above for the final bioassay.

Vitamin D was determined in the feces (or soap suds enema) as follows: The fresh collection was immediately mixed with an equal volume of acetone. The material was then extracted with 250–300 ml. portions of diethyl ether for at least four times. The ether solutions were washed thoroughly with water, shaken with anhydrous Na_2SO_4 and allowed to set with the salt over-night. The filtered ether solutions were concentrated under vacuum. The extract of the stool collected before vita-

[4] Mead Johnson.

min D supplementation was begun was dissolved in ether and distributed directly into the basal ration. The residues of the stools following the vitamin D feeding were dissolved in a known amount of corn oil and stored on ice until bioassayed. The oils were fed to the rats orally by the micro syringe. Although this method of concentrating the vitamin D in human feces was not tested critically it is believed that nearly all of the vitamin was extracted.

The vitamin D in each sample was estimated from the average numerical response of rats fed the reference cod liver oil and also from that of a "master curve" which was made. Recourse to the latter was necessary because this method is essentially one of "trial and error" and the supply of milk was limited. In every case we endeavored to use as many test animals as was practical. The milk samples were stored in the cold and preserved with formalin as prescribed. The average numerical values (arbitrary score) for the degree of cure of the rachitic tibiae in fifteen or sixteen test animals per group were determined.[5] Each group received a total of 32 ml. of a 9% reconstituted skim milk solution and a total of 1, 3, 4, 5 or 7 U.S.P. XI units of vitamin D in the form of Reference Cod Liver Oil. A reading of one represents a single, narrow, continuous black-stained line in the decalcified area (metaphysis) of the halved tibia.[5]

Although the colostra were bioassayed independently, the data are not treated separately because the demarcation of colostrum and milk was not too apparent and, moreover, because the total quantity of the first, watery "milk" usually was enough for one to three rats.

RESULTS

Control milks

From a study of twenty-one control mothers[6] vitamin D could not be detected in the whole milk of eighteen cases when as much as 50–60 ml. of milk were bioassayed. The milks of the remaining three mothers showed 17, 28 and 40 I.U. per liter. Most of the bioassays were done on seven to nine rats and occasionally on less, the average number being 7.0. The volumes of milk consumed per rat were from 13 to 68 ml.,

[5]

U. S. P. XI UNITS VITAMIN D SUPPLIED	NUMERICAL RESPONSE FOR GROUP
1	0.4
3	1.4
4	1.9
5	2.1
7	2.3

[6] Four of these patients, nos. 56, 57, 58 and 59, taken from another experiment, received about 1 gm. of lecithin beginning with lactation. The lecithin, bioassayed at a 0.44 gm. level. had no vitamin D.

the average being 38.6. It was difficult to induce the animals to consume larger quantities of milk. Since 1 unit of vitamin D in Reference Cod Liver Oil gave a healing score of 0.4. i.e., theoretically, $\frac{2}{5}$ of a narrow, continuous line of calcification in the stained metaphysis of the split tibia, it is estimated that about 10 I.U. per liter could be detected. It is concluded, therefore, that the vitamin D of control milks was either absent or less than 10 units per liter. Harris and Bunker ('39) pooled 15 gallons of human milk, collected during the winter and estimated that the amount of vitamin D present was about 4 I.U. (and less than 10 I.U.) per liter. Our control values and in fact all those recorded for "normal" values of vitamin D in mother's milk disagree with the high amounts, namely, an average of 60 I.U. per liter, recorded by the English workers Drummond, Gray and Richardson ('39).[7]

Supplementation during pregnancy

Beginning as early as the fifth month of pregnancy and continuing to term, thirty mothers each received a total of from 32,000 to 480,000 I.U. of vitamin D in the form of fish liver oil. The following groups have been arbitrarily arranged: group I, 32,000 to 95,000 I.U.; group II, 128,000 to 224,000; group III, 256,000 to 320,000; group IV, 416,000 to 480,000 I.U. The results are presented in table 1 and summarized in table 2. The striking finding in these studies is the relatively poor increase of vitamin D in the milk in spite of the large amount of the vitamin in the diet. Seven out of twenty-seven patients receiving from 32,000 to as much as 448,000 units secreted no vitamin D in the milks. The mean vitamin D values obtained in the milks were 10 to 62 I.U. per quart.[8] Large variations were observed among these samples. No relationship was apparent between the amount secreted and the total dose supplied except that the average amount of vitamin D in the milks of mothers fed more than 100,000 units (table 1) before term was significantly larger than those fed from 32,000 to 96,000 I.U. It so happens that with this latter group (table 1) also the largest number of days elapsed between the last dose of 32,000 I.U. and the onset of lactation. However, even among the women fed more than 32,000–96,000

[7] Of the twenty-seven patients tested, only three mothers yielded milks which bioassayed 20 to 35 I.U. per liter; twenty-three, from 40 to 90 I.U. per liter and one case (no. 13) had as much as 180 I.U. per liter. These data are particularly high in view of the fact that the authors stated that the dietary was the sole source of vitamin D and calculated to contain from 35 to 220 I.U. of vitamin D daily.

[8] One case (M, no. 44) is omitted from this series. Following a total intake of 416,000 I.U. units, only a single ounce of "colostrum" plus milk, was collected. Bioassayed with only four rats, it was calculated at 625 I.U. per quart. Unfortunately, this patient had poor breast function and it was impractical to obtain further samples.

TABLE 1

The effect of vitamin D administration during pregnancy on the vitamin D content of human milk.

CASE NO.	PERIOD DURING PREGNANCY WHEN VITAMIN D WAS SUPPLIED	TOTAL VITAMIN D FED	DAYS ELAPSED AFTER LAST VITAMIN D INTAKE AND ONSET OF LABOR	VITAMIN D RECOVERED IN MILK		
	Months	U.S.P. XI units (Each dose equals 32,000 units)		Total Rats	Average ml. milk fed to rat	U.S.P. XI units vitamin D per liter of milk
	I. 32,000 to 96,000 units vitamin D administered during pregnancy					
18-P	6½	32,000	71	11	21	31
19-R	7	32,000	59	7	23	0 to trace
20-D	Labor	32,000	1	10	26	91
21-A	Labor	32,000	1	10	28	216
22-K	5¼ to 5¾	64,000	98	12	31	0 to trace
23-M	7¼ to 7¾	96,000	36	10	40	40
24-N	6¾ to 7½	96,000	40	6	32	0 to trace
25-L	6½ to 7	96,000	49	7	36	0 to trace
26-G	7 to 7½	96,000	45	5	28	0 to trace
	II. 128,000 to 224,000 units vitamin D administered during pregnancy					
27-L	7¼ to 8¾	128,000	6	11	39	32
28-R	7¾ to 8½	160,000	10	11	36	109
29-S	7½ to 8¼	160,000	21	4	40	25
30-A	5¼ to 7¾	160,000	33	13	30	0 to trace
31-B	7¾ to 8¾	160,000	10	6	28	10
32-M	7¼ to 8½	160,000	15	10	32	45
33-V	7½ to 8¾	160,000	9	5	29	93
34-L	7¼ to labor	192,000 (including 32,000 in labor)	1	5	12	148
35-B	7¼ to 8¾	224,000	6	8	31	140
36-M	7 to 8¾	224,000	8	12	22	26
	III. 256,000 to 320,000 units vitamin D administered during pregnancy					
37-M	7 to 8¾	256,000	10	8	38	53
38-S	6½ to 8¾	256,000	6	10	30	23
39-M	6¾ to 8¾	256,000	8	8	21	61
40-W	5¼ to 8½	288,000	12	13	23	156
41-S	5½ to 8½	320,000	13	6 7	8 32	19
	IV. 416,000 to 480,000 units vitamin D administered during pregnancy					
42-T	6 to 9	416,000	7	9 10	32 8	22
43-B	5¾ to 8½	416,000	17	10	4	138
44-M	5¾ to 8¾	416,000	9	4	8	625
45-A	5½ to 8½	416,000	12	5 7	8 24	42
46-G	5½ to 9	448,000	8	10	8	0 to trace
47-S	5 to 8¾	480,000	7	8	24	92
48-B	4½ to 8	480,000	30	5 3	16 4	24

units there is also no apparent influence of the length of the period between the last dose and beginning of lactation.

When the dose was given precisely at labor, the factor of time is significant with reference to the enrichment of the milk. Thus, case D (no. 20) and case A (no. 21) secreted 91 and 216 units, respectively, of vitamin D per quart of milk following a single dose of 32,000 units during labor. Such levels were not realized in eight mothers who received four to fifteen times this dose during pregnancy, the last dose (32,000 units) being administered as long as 10 days before the onset of labor.

TABLE 2

Summary of experimennt.

SUPPLEMENTATION PERIOD OF VITAMIN D	TOTAL CASES	TOTAL INTAKE OF VITAMIN D	VITAMIN D SECRETED PER LITER OF MILK (AVERAGE OF FIRST WEEK'S COLLECTION)			VITAMIN D ABSENT IN MILK
			Lowest value	Highest value	Aver-age	
		U.S.P. XI Units	U.S.P. XI Units			Total cases
0	21 (controls)	Normal diet only	0	40	0 to trace	18
Between 5th and 9th month of pregnancy	7	32,000 to 96,000	0	40	10	5
	9	128,000 to 224,000	0	148	53	1
	5	256,000 to 320,000	19	156	62	0
	6	416,000 to 480,000	0	138	53	1
From 7th month to term	1	192,000 (including 32,000 during labor	.. ..	.. ..	148	..
During Labor only (24 hours)	2	32,000	91	216	153	0
During Labor to first 9 days after delivery	7	320.000 to 400,000	125	583	310	0

Patient L (no. 34), who received a total of 192,000 I.U. of vitamin D between the seventh month to term, including 32,000 units during labor, produced a milk containing 148 I.U. per quart. It is our opinion that the last dose supplied in labor was largely responsible for the quantity recovered.

Post-natal supplementation of vitamin D

Seven patients were given daily doses of 40,000 I.U. of vitamin D by capsule beginning during labor and continuing through the first 7 to 9 days of lactation. The capsule was fed about 6 P. M., and the milk samples were collected about 15 hours later. As seen in table 3 these patients secreted milks containing from 125 to 583 I.U. of vitamin D per quart. The mean value of 310 I.U. corresponded to a mean intake of 348,000 units in the form of fish liver oil. Although the average quantity recovered was low, the mean value is five to six times higher than the means for mothers supplied the vitamin during pregnancy only (table 2).

TABLE 3

Group V which received 320,000 to 400,000 I.U. of vitamin D beginning with labor and continuing during lactation.

CASE NO.	TOTAL VITAMIN D FED	VITAMIN D RECOVERED IN MILK		
	U.S.P. XI Units (1 dose equals 40,000 units)	Total rats	Total ml. milk fed to rat during bioassay	U.S.P. IX Units vitamin D per liter of milk
49-M	320,000	8 11	32 16	125
50-G	320,000	7 11	32 16	250
51-W	360,000	9	16	312
52-N	360,000	10	16	156
53-S	360,000	8	16	312
54-B	400.000	9	16	438
55-G	320,000	14 9	12 6	583

Balance study with patient G, no. 55

On a 23-year-old negro patient (G, no. 55) of the last group described vitamin D was determined in the milk, blood and stools. Following a daily intake of 40,000 I.U. of vitamin D for 8 days, beginning with

labor, the amount recovered in the milk when bioassayed at 1.5 and 0.75 ml. levels, was 666 and 500 I.U. per quart. Blood samples, taken approximately 14 hours after the dose on the fifth and ninth days, contained 393 and 384 I.U. per 100 ml., respectively, as compared with the 118 I.U. found in the pre-test specimen. (Warkany and Mahon, '40, bioassayed sera of 155 normal humans and reported a range from 66 to 165 I.U. per 100 ml.) The pre-test stool contained no vitamin D; those collected as quantitatively as practical until the end of the ninth day, totalled 22,820 I.U. of vitamin D. The data are summarized in table 4.

We observed the following recovery values of the ingested vitamin D for case G (no. 55):

TABLE 4

Balance study of vitamin D in case no. 55 (secundipara; colored; 60 kg.; age 23 years).

POST-PARTUM DATE	GIVEN DAILY BY CAPSULE 40,000 U.S.P. XI UNITS VITAMIN D	U.S.P. XI UNITS VITAMIN D RECOVERED					
		Milk		Blood		Stool	
		Units per liter	Rats tested	Units per liter	Rats tested	Units per pooled output	Rats tested
9/23/42 A. M.	(labor)-0			1180	3	0	4
9/23 6 P. M.	40,000						
9/24	40,000					2900	10
9/25	40,000						
9/26	40,000					4250	10
9/27	40,000	666	14	3930[1]	12		
9/28	40,000	and				10310	10
9/29	40,000	583	9	(9/27/42)			
9/30	40,000			3850[1]	12	4820	11
10/1	0			(10/1/42)			

[1] About 14 hours after capsule was given.

(a) In milk, 1.3%, assuming 1 quart is secreted in 24 hours and deducting the amount given by a theoretical pre-test milk having a liberal level of 50 I.U. per quart.

(b) In stools, 6.9%, assuming that this is the total which was not absorbed, that no changes (due to bacterial effects) occurred in passage through the tract, and that the blood contributed none of this amount.

(c) In the blood about 14 hours after the dose, 36.5%; assuming 90 ml. of blood per kilogram body weight (Best and Taylor, '39) and deducting the quantity found in the pre-test sample.

From these values, it is obvious that a large part of the ingested vitamin D is unaccounted for.

DISCUSSION OF RESULTS

After reviewing papers reporting tests where mothers were fed vitamin D in the form of fish liver oil during pregnancy [9] it was concluded that perhaps larger amounts than those fed are necessary to increase the content of vitamin D in human milk. We supplied, therefore, significantly higher levels of the vitamin during pregnancy, and were not successful in seven out of twenty-seven cases. Milks were enriched with vitamin D in every case when doses of 32,000 to 40,000 I.U. were given during labor or thereafter. As early as 1924, Hess and Weinstock observed rickets in fifteen out of twenty-eight babies born of mothers who received a total of nearly 16 ounces (estimated to be about 37,000 I.U.) of cod liver oil in the eighth and ninth months of pregnancy. Moreover, when 1000 I.U. of vitamin D as halibut liver oil were supplied daily to six English mothers during the latter half of pregnancy, the subsequent milks were not appreciably richer in the factor than those of control subjects (Drummond, Gray and Richardson, '39). Apparently, the capacity to transfer the vitamin D of fish liver oil to breast milk is quite limited. Nevertheless, the possible beneficial effects on both mother and fetus, e.g., improvement in the Ca and P retention, should not be overlooked (Macy et al., '30; Liu and coworkers, '41). In our study with patient G (no. 55) who ingested 40,000 I.U. of the vitamin daily beginning with the onset of labor, it was found that approximately 1.3% of the ingested vitamin was accounted for in the milk; and in two patients (D, no. 20 and A, no. 21), fed a single dose of 32,000 I.U. during labor only, about 0.3 and 0.7% was similarly secreted.

Seven references were found in the literature to studies in which attempts were made to increase the vitamin D level of cows' milk by feeding fish liver oil specifically.

The early workers (Lesne and Vagliano, '24; Wagner and Wimberger, '25; Golding and his coworkers, '26, '28; Both, '34) did succeed in increasing somewhat (units omitted) the vitamin D of cow's milk by feeding cod liver oil. Steenbock et al. ('30) supplied a cow daily for over 2 years, 180 gm. of cod liver oil (about 9700 I.U.) and found that the antirachitic properties of her milk was not improved over that yielded by control cows. By employing daily massive doses, Krauss, Bethke and Wilder ('33) were more successful in raising the vitamin D content of the butterfat. We calculated from their data a recovery of about 0.8 to 1.6% of the ingested vitamin D in the milk.

[9] A bibliography will be submitted on request.

It is apparent that vitamin D must be available beyond the normal intakes just prior to lactation in order to enrich the milk with the factor. Eufinger, Wiesbader and Focsaneanu ('29) concluded that the transfer of vitamin D to the milk is an immediate one because rachitic rats were healed when fed milk from mothers given 10 drops of vigantol 1 day only before lactation. These investigators found that the milk from mothers fed 10 drops vigantol (irradiated ergosterol in oil) several months before delivery also prevented rickets in rats, in contrast to colostrum or milk from control mothers fed ad libitum in the prophylactic bioassay. Unfortunately, these milks were not bioassayed quantitatively. McCollum et al. ('27) stated that young rats were afforded a much higher degree of protection against rickets when the cod liver oil was supplied to the mother during lactation only than during pregnancy only. Other attempts to fortify human breast milk by supplying the vitamin during lactation are noteworthy. Gerstenberger and Russell ('30) were not successful in curing rachitic infants with the milk from lactating mothers fed 15 ml. (about 1170 I.U.) of cod liver oil daily (test period not stated), nor with the milk from those on an already adequate diet further supplemented daily with 1 tablespoonful of cod liver oil (250 I.U.) for 45 days (Gerstenberger, Hartman and Smith, '27). Weech ('27) concluded from his studies that a certain amount of the antirachitic vitamin does pass into the mother's milk because the degree of rickets in his series of babies was, in general, inversely proportional to the previous intake of oil by the nursing subject. Wide variations were encountered, however.

The fate of vitamin D in the body

Our knowledge concerning the fate of vitamin D in the body is still unsatisfactory. On the assumption that the amount of vitamin D which appeared in the feces of patient G (no. 55) represented that quantity (7%) which escaped absorption, it is concluded that the mixed fish liver oil was absorbed very efficiently. Only three papers could be found in the literature reporting bioassays of human feces for vitamin D. Hess, Weinstock and Gross ('33) and Hess ('33) bioassayed stools of infants fed viosterol, cod liver oil, and "vitamin D" milks and concluded that the amount of vitamin D excreted was dependent on the intake, absolute units were not reported. Windorfer ('38) recovered 3 to 14% (average 7%) of the vitamin D fed to rachitic infants in these fecal extracts. Using dogs, Windorfer recovered from 1 to 29% (average 20% of the ingested vitamin D after 48 hours.

Heymann ('37, '38) supplied adult dogs with a single dose of 250,000 U.S.P. units of vitamin D in the form of viosterol in oil or crystalline D_2 in propylene glycol and was able to find appreciable quantities excreted in the stool 6 to 8 months after the supplement was given. Morgan and Shimotori ('43) using essentially the Heymann ('37) method found little vitamin D on the first day and none thereafter, in the stools of three young dogs (50–85 kg.) which were fed a single massive dose of 20,000 I.U. of vitamin D per kilogram body weight in the form of tuna liver oil, irradiated ergosterol and delsterol (i.e., irradiated cholesterol).

It appears from these studies that man and dog absorb vitamin D very well. However, from studies with the chick (Klein and Russell, '31) and the cow (Hart et al., '29, '30; Hess et al., '32; Krauss, Bethke and Wilder, '34) it is agreed that a relatively huge amount of the ingested vitamin D appears in the feces. It is interesting to point out, moreover, that the associate fat soluble vitamin, namely, vitamin A, is absorbed to the extent of 90% in the hen (Russell et al., '42), rat (Vedder and Rosenberg, '38; De, '37; Baumann and coworkers, '34; Davies and Moore, '34), cat (Ahmad, '31), infant and adult humans maintained on various diets (Rowntree, '30; Anderson, '38, '39; Wilson, Das Gupta and Ahmad, '37; Catel, '38).

Although the urine of patient G (no. 55) was not bioassayed for vitamin D, it is of interest that Hess et al. ('33) found no antirachitic activity in 50 ml. of urine from a cow fed daily 270,000 I.U. of vitamin D as irradiated yeast. Moreover, Lawrie and his coworkers ('41) found no vitamin D in the urine of a normal human (sex not stated) 24 hours following a dose of 9000 I.U. in the form of calciferol, nor in the urine of a dog fed 20,000 I.U. similarly. No vitamin A was recovered in the urines of pregnant women, nor in two given 12,000 I.U. of vitamin A (as halibut oil) for 1 month or 70,000 I.U. daily for 1 week. These values for the vitamin A in urines of pregnant women are much less than those reported by Gaehtgens ('37) for normal pregnant human subjects.

The failure to find appreciable amounts of vitamin D in the milks of mothers fed this supplement prior to labor may be due to the inactivation of this factor. It is agreed in the literature that the larger proportion of the ingested vitamin D is presumably destroyed or converted to some inactive material. Light, Wilson and Frey ('34) concluded that vitamin D, unlike vitamin A, is not stored in the liver of this species since "livers of cows which for months have been receiving approximately 70,000 units (189,000 I.U.) of vitamin D in excess of the amount secreted in the milk or excreted in the feces have only one unit (2.7 I.U.)

of vitamin D per gram of fresh liver at the end of this time.'' Guerrant et al. ('38) fed calves cod liver oil and irradiated yeast for as long as 7 months and are in agreement with the above workers. Heymann ('37) using rabbits found that various tissues retained vitamin D for several weeks following a single dose of 200,000 U.S.P. units of viosterol in oil. Likewise, Morgan and Shimotori ('43) fed dogs 200,000 I.U. per kilogram body weight accounted for less than 10% of the ingested vitamin D in the tissues. Similar findings were reported by Remp ('41) using rachitic rats. Following a single dose of 100 I.U. of crystalline vitamin D_2, from $\frac{1}{3}$ to $\frac{1}{4}$ of the amount administered remained in the body of the rat at the end of 24 hours. After 10 days, only 5 to 10% was found in the body. Small and relatively constant amounts of the vitamin (quantity not stated) were observed in the feces during these periods.

Studies of vitamin D retention in human tissues have been done on only four children to date, these being given large amounts of vitamin D from 36 hours to 26 days before exitus (Vollmer, '39, '41; and Windorfer, '38). In spite of the massive quantities (over a million units) supplied by Vollmer to three of these cases by intramuscular injection, the recovery of the vitamin in the tissues studied was inappreciable. Windorfer ('38) fed 15 mg. (600,000 I.U.) of vitamin D_2 (irradiated ergosterol) to a baby with pneumonia 2 weeks before death and found only 3.3% in the brain, 4% in the kidney, and traces in liver and intestinal tissues using the colorimetric procedure of Brockman.

Although it is known that the concentration of vitamin D in the blood stream governs the amount secreted in the milk (cow's), the processes involved are a matter of speculation. Thus far, for humans, we do not know the limits of this secretion, nor the minimal amount of a given source of the vitamin which will just raise the vitamin D level of human milk.

That the medium containing the vitamin plays a significant role in the effectiveness of vitamin D has been well established (Lewis, '35, '36; Greaves and Schmidt, ''34; Shelling, '37; Supplee et al. '36). Hess, Lewis, McLeod and Thomas ('31) found that irradiated yeast induced a more potent milk per unit of vitamin D fed than viosterol. Russell et al. ('34) successfully increased the vitamin D level of cow's milk beyond that incurred by the feeding of irradiated ergosterol in oil when 0.75% hydroquinone was added. Thus the problem of increasing the vitamin D content of the milk as secreted still remains an open field for further study.

SUMMARY

1. The vitamin D level in the milks of eighteen mothers who received no preformed sources of the factor in the dietary during pregnancy was inappreciable, ranging from traces to less than 10 I.U. per quart. Three cases showed values of 17, 28 and 40 I.U. per quart.

2. The average vitamin D content per quart of milk of mother supplied during the latter half of pregnancy was as follows:

(a) 10 I.U. for seven mothers given from 32,000 to 96,000 I.U. of supplement.

(b) 53 I. U. for nine mothers given from 128,000 to 224,000 I.U. of supplement.

(c) 62 I.U. for five mothers given from 256,000 to 320,000 I.U. of supplement.

(d) 53 I.U. for six mothers given from 416,000 to 480,000 I.U. of supplement.

3. Two mothers given a single dose of 32,000 I.U. during labor secreted milks from 3 to 8 days later, which bioassayed 91 and 216 I.U. per quart (0.3 and 0.7%, respectively, of calculated recovery). Seven women supplied 40,000 I.U. of vitamin D daily from labor to about 10 days later yielded milks, the vitamin D content of which averaged 310 I.U. per quart (range 125 to 583 I.U.).

4. It is concluded, therefore, that for the purpose of fortifying human milk with vitamin D for infant use, supplementation of the mother with fish liver oil during pregnancy is not a practical method. Other beneficial effects on mother and fetus are not overlooked, however.

Vitamin D must be taken just prior to or during lactation in order to enrich the milk with the factor appreciably.

5. The minimal dose of vitamin D which, when added as a supplement to the mother's basal diet, will yield a satisfactory increase of vitamin D in her milk has not been determined.

6. In an 8-day balance study a patient who received 40,000 I.U. daily in the latter group secreted about 1.3% of the ingested vitamin D in the milk and 6.9% in the stools. Approximately 36% of the factor was accounted for in blood samples taken about 14 hours after the dose. It is presumed that the vitamin D of fish liver oil was very efficiently absorbed, the larger proportion, however, having been inactivated or destroyed very rapidly.

LITERATURE CITED

AHMAD, B. 1931 The fate of carotene after absorption in the animal organism. Biochem. J., vol. 25, p. 1195.

ANDERSEN, O. 1938 The effect of the administration of paraffin oil on the resorption of vitamin A in man. Hospitalstid. (Supp., Festskr. Bisp. Hosp.), vol. 81, p. 29.

——— 1939 Untersuchungen über die Wirkung von Paraffinöleingabe auf die Resorption des A-Vitamins bei Menschen. Klin. Wochenschr., Bd. 18, p. 499.

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

BECHTEL, H. E., AND C. A. HOPPERT 1936 A study of the seasonal variation of vitamin D in normal cow's milk. J. Nutrition, vol. 11, p. 537.

BEST, C. H., AND N. B. TAYLOR 1939 Physiological basis of medical practice. Second edition. Williams and Wilkins Co., Baltimore, Md.

BOTH. R. G. 1934 Ph. D. Thesis. Cited by A. T. R. Mattick, 1937: "The handling of milk and milk products." Ministry of Agriculture and Fisheries, London, Bull. no. 37, p. 97.

CATEL, W. 1938 Klinische und tierexperimentelle Studien über die normale und pathologische Physiologie des A-Vitamins. Monatschr. für Kinderh., Bd. 73, S. 316.

COOPER, M. L. AND F. J. GRABILL 1939 A simplified apparatus for the preservation of bacterial cultures in the dried state. J. Lab. Clin. Med., vol. 25, p. 184.

DAVIES, A. W., AND T. MOORE 1934 Distribution of vitamin A in organs of normal and hypervitaminotic rat. Biochem. J., vol. 28, p. 288.

DE, N. K. 1937 Assimilation of vitamin A and carotene by rats from some common foods with note on conversion factor I.V./E. proposed by International Vitamin Conference. Indian J. Med. Research, vol. 24, p. 751.

DRUMMOND, J. C., C. H. GRAY AND N. E. G. RICHARDSON 1939 The antirachitic value of human milk. Brit. Med. J.. vol. 2, p. 757.

EUFINGER, H.. H. WIESBADER AND L. FOCSANEANU 1929 Tierexperimentelle Studien zur Vigantolprophylaxe. Klin. Wochenschr., Bd. 8, S. 826.

GAEHTGENS, G. 1937 Über die Ausscheidung von Vitamin A in der Gravidität. Klin. Wochenschr., Bd. 16, S. 52.

GERSTENBERGER, H. J., J. I. HARTMAN AND D. N. SMITH 1927 The antirachitic value of human milk. Calif. and Western Med., vol. 27, p. 40.

GERSTENBERGER, H. J., AND G. R. RUSSELL 1930 Studies on wet nurses receiving irradiated ergosterol. Acta Paediat., vol. 11, p. 32.

GREAVES, J. D., AND C. L. A. SCHMIDT 1934 The action of viosterol in jaundiced rachitic rats. Univ. of Calif. Publ., Physiol., no. 6, vol. 8, p. 49.

GUERRANT. N. B., R. A. MORCK, S. I. BECHDEL AND N. W. HILSTON 1938 The storage of vitamin D in the tissues of growing calves. Proc. Soc. Exp. Biol. Med., vol. 38, p. 827.

HARRIS, R. S., AND J. W. M. BUNKER 1939 The vitamin D potency of human breast milk. Am. J. Pub. Health, vol. 29, p. 744.

HART, E. B., H. STEENBOCK, O. L. KLINE AND G. C. HUMPHREY 1930 Dietary factors influencing calcium assimilation. XIII. The influence of irradiated yeast on the calcium and phosphorus metabolism of milking cows. J. Biol. Chem., vol. 86, p. 145.

HART, E. B., H. STEENBOCK, E. C. TEUT AND G. C. HUMPHREY 1929 Dietary factors influencing calcium assimilation. The influence of cod-liver oil upon the calcium metabolism of milking cows. J. Biol. Chem., vol. 84, p. 359.

HESS, A. F. 1933 The fate of various antirachitics within the body. Acta Paediat., vol. 16, p. 467.

HESS, A. F., J. M. LEWIS, F. L. MACLEOD AND B. H. THOMAS 1931 Antirachitic potency of the milk of cows fed irradiated yeast or ergosterol. A clinical test. J. Am. Med. Assn., vol. 97, p. 370.

HESS, A. F., AND M. WEINSTOCK 1924 Rickets as influenced by the diet of the mother during pregnancy and lactation. J. Am. Med. Assn., vol. 83, p. 1558.

Hess, A. F., R. F. Light, C. N. Frey and J. Gross 1932 A study of the milk, blood, and excreta of cows fed moderate and excessive amounts of irradiated yeast or ergosterol. J. Biol. Chem., vol. 97, p. 369.

Hess, A. F., M. Weinstock and J. Gross 1933 A biologic assay of blood and feces of infants receiving various antirachitics. Proc. Soc. Exp. Biol. Med., vol. 30, p. 1357.

Heymann, W. 1937 Metabolism and mode of action of vitamin D. II. J. Biol. Chem., vol. 118, p. 371.

——— 1937–38 Metabolism and mode of action of vitamin D. IV. J. Biol. Chem., vol. 122, p. 249.

Klein, D., and W. C. Russell 1931 The fate of the antirachitic factor in the chicken. The antirachitic factor balance in the growing chick. J. Biol. Chem., vol. 93, p. 693.

Krauss, W. E., R. M. Bethke and W. Wilder 1933 The effect of feeding a cod liver oil concentrate to cows on the vitamin D content of milk. J. Dairy Sci., vol. 16, p. 549.

——— 1934 The relative efficiencies of irradiated ergosterol and irradiated yeast for the production of vitamin D milk. J. Dairy Sci., vol. 17, p. 685.

Lawrie, N. R., T. Moore and K. J. Rajagopal 1941 The excretion of vitamin A in urine. Biochem. J., vol. 35, p. 825.

Lesné, E., and M. Vagliano 1924 De l'influence du lait de femme sur le rachitisme experimental. Compt. Rend. Soc. Biol., vol. 91, p. 143.

Lewis, J. M. 1935 Clinical experience with crystalline vitamin D: The influence of menstruum on the effectiveness of the antirachitic factor. J. Pediat., vol. 6, p. 362.

——— 1936 Further observations on the comparative antirachitic value of crystalline vitamin D administered in milk, corn oil, or in propylene glycol. J. Pediat., vol. 8, p. 308.

Light, R. F., L. T. Wilson and C. N. Frey 1934 Vitamin D in blood and milk of cows fed irradiated yeast. J. Nutrition, vol. 8, p. 105.

Liu, S. H., H. I. Chu, C. C. Su, T. F. Yu and T. Y. Cheng 1940 Calcium and phosphorus metabolism in osteomalacia IX. Metabolic behavior of infants fed on breast milk from mothers showing various states of vitamin D nutrition. J. Clin. Inv., vol. 19, p. 327.

McCollum, E .V., N. Simmonds, J. E. Becker and P. G. Shipley 1927 Studies on experimental rickets. XXVIII. Does vitamin D pass into the milk? Am. J. Dis. Child., vol. 33, p. 230.

Macy, I. G., H. A. Hunscher, B. Nims and S. S. McCosh 1930 Metabolism of women during reproductive cycle. Calcium, phosphorus, and nitrogen utilization in lactation before and after supplementing the usual home diets with cod liver oil and yeast. J. Biol. Chem., vol. 86, p. 59.

Morgan, A. F., and N. Shimotori 1943 The absorption and retention by dogs of single massive doses of various forms of vitamin D. J. Biol. Chem., vol. 147, p. 189.

Natelson, S., and A. E. Sobel 1935 A new method for the separation of sterols from vitamin D-containing materials. J. Biol. Chem., vol. 109, p. 687.

Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists 1940 The Collegiate Press, George Banta Publishing Company. Menasha, Wisconsin.

Remp, D. G. 1941 Storage and excretion of vitamin D in the rat after a single oral dose. J. Biol. Chem., vol. 140, Scientific Proceedings, Soc. of Biol. Chemists, p. CV.

Rowntree, J. I. 1930 A study of the absorption and retention of vitamin A in young children. J. Nutrition, vol. 3, p. 265.

Russell, W. C., M. W. Taylor, H. A. Walker and L. J. Polskin 1942 The absorption and retention of carotene and vitamin A by hens on normal and low fat rations. J. Nutrition, vol. 24, p. 199.

RUSSELL, W. C., D. E. WILCOX. J. WADDELL AND L. T. WILSON 1934 The relative value of irradiated yeast and irradiated ergosterol in the production of vitamin D milk. J. Dairy Sci., vol. 17, p. 445.

SHELLING, D. H. 1937 Calcium and phosphorus studies. The effect of emulsification on the potency of viosterol in the treatment of rickets in children. J. Pediat., vol. 10, p. 748.

SOBEL, A. E., A. R. GOLDFARB AND B. KRAMER 1935 Studies of incurable rickets. II. Role of the "local factor" and of viosterol in the pathogenesis of rickets due to beryllium. J. Biol. Chem., vol. 108, p. 395.

STEENBOCK, H., E. B. HART, F. HANNING AND G. C. HUMPHREY 1930 Fat-soluble vitamins. The anti-rachitic value of cow's milk as modified by feeding of irradiated yeast. J. Biol. Chem., vol. 88, p. 197.

SUPPLEE, G. C., S. ANSBACHER, R. C. BENDER AND G. E. FLANIGAN 1936 The influence of milk constituents on the effectiveness of vitamin D. J. Biol. Chem., vol. 114, p. 95.

TAYLOR, M. W., D. KLEIN AND W. C. RUSSELL 1938 Photographing line tests in vitamin D assays. Ind. Eng. Chem., Anal. Ed., vol. 10, p. 26.

VAN NIEKERK, J. 1933 Quantitative data on the content of antirachitic vitamin in cow's milk and mother's milk. Maandschr. v. Kindergeneesk., vol. 2, p. 525.

VEDDER, E. B., AND C. ROSENBERG 1938 Concerning toxicity of vitamin A. J. Nutrition, vol. 16, p. 57.

VOLLMER, H. 1939 Distribution of vitamin D in the body after administration of massive doses. Am. J. Dis. Child., vol. 57, p. 343.

——— 1941 Distribution of vitamin D in brain after repeated administration of massive doses; histologic investigation of D-hypervitaminosis. Arch. Pediat., vol. 58, p. 9.

WAGNER, R., AND H. WIMBERGER 1925 Über den Einfluss von Lebertranverfutterung. Zeit. für Kinderheilk. Bd. 40, S. 295.

WARKANY, J., G. M. GUEST AND F. J. GRABILL 1942 Estimation of vitamin D in blood serum III. Vitamin D in human serum during and after periods of ingestion of large doses of vitamin D. J. Lab. and Clin. Med., vol. 27, p. 557.

WARKANY, J., AND H. E. MAHON 1940 Estimation of vitamin D in blood serum II. Level of vitamin D in human blood serums. Am. J. Dis. Child., vol. 60, p. 606.

WEECH, A. A. 1927 The influence of the administration of cod liver oil to the mother on the development of rickets in the infant. Bull. Johns Hopkins Hosp., vol. 40, p. 244.

WILSON, H. E. C., S. M. DAS GUPTA AND B. AHMAD 1937 Studies on the absorption of carotene and vitamin A in human subjects. Indian J. Med. Research, vol. 24, p. 807.

WINDORFER, A. 1938 Über die Vitamin-D-Resorption bei Vera-Breichung hoher dosen (Vitamin D-Stoss). Klin. Wochenschr., Bd. 17, S. 228.

VITAMIN A REQUIREMENTS IN CALVES[1]

J. M. LEWIS AND LOGAN T. WILSON

Department of Pediatrics, New York University College of Medicine; Department of Laboratories, Beth Israel Hospital, New York City and The Walker Gordon Laboratories, Plainsboro, New Jersey

TWO FIGURES

(Received for publication July 12, 1945)

In previous communications (Lewis et al., '39, '42) we have reported that the minimum vitamin A requirements in infants are of the same order of magnitude as in rats, namely, 20 to 35 U.S.P. units per kilogram of body weight. This level of vitamin A intake is adequate for fairly good growth and prevents the development of xeropthalmia and other gross manifestations of vitamin A deficiency. However, in order to obtain optimal blood levels of vitamin A and good storage in the liver, the requirements for vitamin A are increased several fold.

The present study was undertaken to obtain information on the relationship of vitamin A intake to growth, blood concentration of vitamin A and liver storage in young calves.

PLAN OF EXPERIMENT

Twenty-six Grade Holstein calves, male and female, 2 to 9 days old were placed on a diet nearly devoid of vitamin A in order to deplete their stores of this vitamin. At first they were fed skimmed milk and when they were able to ingest solid food, they were given the following ration: ground barley 27 parts, linseed oil meal 23 parts, wheat middlings 11.5 parts, oat mill feed 40 parts, ground limestone 3 parts, salt 0.5 parts and irradiated yeast 0.1 parts. This low vitamin A ration is a modification of the one described by Boyer and his co-workers ('42). Skimmed milk was discontinued when the calves were 16 weeks of age. The calves were housed in a separate building and were allowed to run in dry lots when the weather was favorable.

The animals were weighed twice a week and when a cessation in gain in weight occurred they were divided into six groups, each group consisting of four calves. Thirty-two, 64, 128, 256, 512 and 1024 U.S.P. units

[1] This study was aided by a grant from the Borden Company.

of vitamin A per kilogram body weight were fed to each of these groups, respectively. The daily dose of vitamin A was adjusted weekly to the weight of each calf and was given in a capsule. The vitamin A supplement was obtained from shark liver oil and contained 50,000 units of vitamin A per gram. The animals on the lowest vitamin intake were tested for night blindness throughout the entire experiment.

Blood samples for vitamin A determination were taken from each calf at the end of the depletion period and at approximately monthly intervals while receiving the vitamin A supplement.

Two calves in each group received the supplement for 4 and 8 months, respectively, and were then sacrificed. Vitamin A analyses were carried out on the livers and histological examination of the various organs were made on those animals receiving the smallest amounts of vitamin A in order to determine whether there were any histological evidences of vitamin A deficiency.

The methods of determining the vitamin A in blood and liver were based on the Carr-Price reaction and have been the same as were used in previous investigations (Lewis et al., '39, '42).

For the most part, the calves appeared healthy and continued to grow after the vitamin A supplement feeding was started. The chief difficulty experienced was a low grade type of pneumonia, which a number of the calves contracted while being depleted. The first two calves to show signs of depletion died of pneumonia. Two calves started in group I died after 2 and 3 weeks on supplement, and one in group V after 2 weeks on supplement. All of these calves were replaced and their records do not show in the data reported. Four calves, no. 415, group I; no. 416, group II; no. 427, group III and no. 431, group V, showed some evidence of pneumonia but survived after treatment with sulfapyradine. Calf no. 431 was treated for pneumonia on three different occasions, and it is questionable whether the data on this calf should be included in the averages for the group. For this reason, the data on calf no. 431 were eliminated in the computation of the averages of the gain in weight and blood levels in group V. Of the twenty four calves which were killed at the completion of their feeding period, thirteen showed some post-mortem evidence of having had pneumonia. Some of the calves developed diarrhea during the depletion but this symptom disappeared quite promptly after vitamin A supplement was given.

RESULTS

A comparison of the rate of growth of the various groups of calves may be observed in figure 1. It will be noted that there was no signifi-

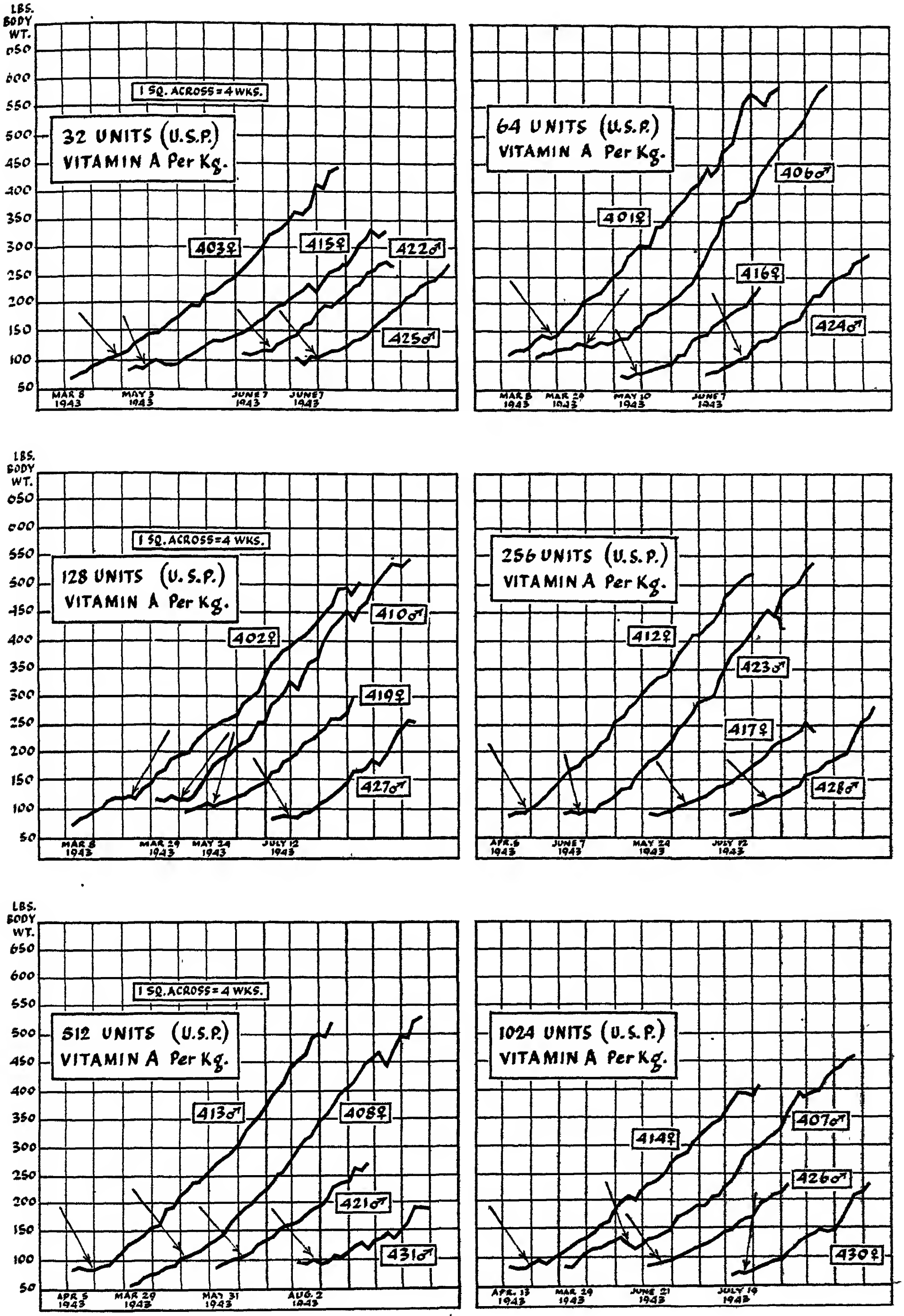


Fig. 1 Growth curves of groups of calves fed various amounts of vitamin A.

cant difference in the rate of gain of weight among the various groups of calves receiving the vitamin A supplements for 4 months. On the other hand, calves receiving 32 units of vitamin A per kilogram body weight and those receiving 1024 units per kilogram body weight for 8 months did not gain as well as those fed 64, 128, 256 and 512 units for the same period.

The average daily rate of gain of all the groups, irrespective of whether the supplement was given for 4 or 8 months, was computed.

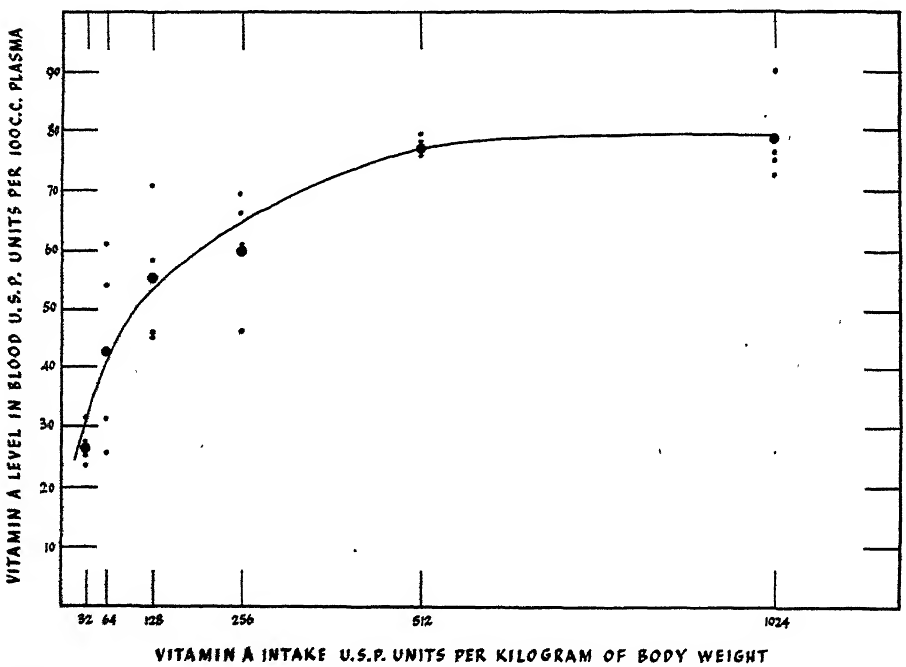


Fig. 2 Relation of vitamin A intake to the concentration of vitamin A in blood plasma.

The average daily rate of gain in group I was 1.23 lbs., in group II, 1.60, in group III, 1.54, in group IV, 1.60, in group V, 1.64 and in group VI, 1.33.

These results would seem to indicate that an intake of 32 units per kilogram of body weight was not quite sufficient for maximal gain in weight over a prolonged period and that, 64 units per kilogram of body weight represented in the minimum vitamin A intake necessary to bring about optimal gain in weight under the conditions of this experiment. It is a matter of speculation whether the slowing of the rate of gain in animals receiving 1024 units per kilogram of body weight was due to a deleterious effect of this large intake of vitamin A on the rate of growth

or was merely coincidental. Additional data would be required to clear up this question.

The relationship of the vitamin A intake to the concentration of vitamin A in the blood is shown in figure 2. It will be observed that there was a direct correlation between the vitamin A intake and the blood level until 512 units per kilogram of body weight were given, at which intake, maximal concentrations of vitamin A were obtained. Thus, (table 1) the average vitamin A concentration in calves of group I was 27 units per 100 ml. of plasma, in group II, 42 units, in group III, 55 units, in group IV, 60 units, in group V, 77 units and in group VI, 78 units.

TABLE 1

Vitamin A concentration in the blood of calves receiving various intakes of vitamin A.

GROUP	VITAMIN A INTAKE PER KILOGRAM BODY WEIGHT (U.S.P. UNITS)	NO. OF CALVES	VITAMIN A LEVEL PER 100 ML. BLOOD PLASMA (U.S.P. UNITS)	
			Average	Range
1	32	4	27	24–27
2	64	4	42	26–61
3	128	4	55	45–71
4	256	4	60	46–69
5	512	3	77	76–79
6	1,024	4	78	73–90

In table 2 the relationship of vitamin A intake to storage in the liver is recorded. It will be noted that there is considerable variation in the degree of storage among animals receiving the same intake of vitamin A. Of seven animals receiving either 32 or 64 units per kilogram of body weight four had no storage and the remaining three had slight to moderate amounts of vitamin A in their livers. In the group of animals receiving 128 units per kilogram of body weight, all four animals had some storage of vitamin A although the amounts were rather small. On the other hand, moderate stores of vitamin A were observed in the livers of most animals receiving 256 and 512 units, and large amounts of vitamin A were found in three of the four animals receiving 1024 units.

DISCUSSION

The results of this experiment indicate that an intake of 32 units of vitamin A per kilogram body weight just about covers the minimum requirement for young calves. At this level of feeding the calves grew fairly well although not to the same extent as those receiving larger amounts of vitamin A. There was no evidence of night blindness in

three of the four calves in this group and histological examination revealed none of the characteristic changes of vitamin A deficiency, namely, metamorphosis of the epithelium into keratinizing stratified squamous epithelium. These observations on the minimum vitamin A requirement of calves are in agreement with those of Guilbert, Miller and Hughes ('37) who found that 6 to 8 µg. of vitamin A (24 to 32 U.S.P. units) per kg. per day represent the minimum requirements for cattle, swine and sheep.

TABLE 2

Relationship of vitamin A intake to liver storage.

VITAMIN A INTAKE PER KILOGRAM OF BODY WEIGHT	CALF NO.	VITAMIN A UNITS PER 100 GM. OF LIVER	VITAMIN A UNITS IN THE WHOLE LIVER
32	403	0	0
	415	640	17,433
	422	0	0
	425	1,600	31,088
64	401	150	6,129
	406	0	0
	424	0	0
128	410	280	15,890
	402	580	18,432
	419	620	11,959
	427	580	13,821
256	412	1,260	45,343
	423	1,000	47,670
	428	3,840	78,450
512	413	1,390	64,676
	408	2,620	95,158
	421	440	11,479
1,024	414	1,650	69,283
	407	12,600	457,632
	426	7,300	132,568
	430	210	4,546

The amount of vitamin A required for optional growth was 64 units per kilo whereas an intake of 512 units was necessary to bring about maximal concentrations of vitamin A in the blood plasma. Moderate storage of vitamin A in the liver resulted in those calves receiving 256 and 512 units per kilo and considerable amounts of vitamin A were obtained in the group receiving the highest intake of vitamin A, namely 1024 units.

Boyer and his co-workers ('42) report a vitamin A intake of 12 µg. per kilogram to be border line and 18 µg. as adequate in young calves.

Assuming that 1 µg. of vitamin A is equivalent to 4 U.S.P. units, the adequate level of 18 µg. corresponds quite well with the level of 64 units fed to group II which we found to be adequate for maximum growth. However, our results show that these intakes of vitamin A will not provide for maximal blood levels or significant storage in the liver.

From a practical standpoint it would seem desirable to feed calves a level of vitamin A which would allow for at least a moderate degree of storage. The results reported herein indicate an intake of 250 U.S.P. units per kilogram or 11,000 units per 100 pounds of live weight is required for this purpose.

The observations in this investigation are of special interest when compared with those reported by Lewis and his co-workers ('39, '42) in their experiments on the vitamin A requirements in infants and in rats. They found that the minimum requirements in infants as judged by dark adaptation was of the same order of magnitude as in rats, namely, 20 to 35 units per kilogram body weight. At this intake the infants grew fairly well and appeared normal in every way. However, the vitamin A concentration in the blood, at this level of intake, was found to be abnormally low. Maximal blood concentrations were obtained in infants as in young rats when the vitamin A intake was approximately twenty-five times the minimum requirements. In their experiments on young rats a fair degree of storage in liver occurred when the feeding was twenty-five times the minimum requirement and good reserves were noted in the livers when the intake was fifty times the minimum requirements.

The data reported in the present communication indicate that in young calves maximal blood levels of vitamin A occurred with an intake of sixteen times the minimum requirement. The storage in the liver was moderate when the amount of vitamin A fed was eight to sixteen times the minimum requirement; large stores in the liver were noted when the intake was thirty-two times the minimum requirement. Thus, in general, the same situation occurs in calves as in rats and in infants with respect to the relationship of vitamin A intake to growth, concentration of vitamin A in the blood and liver storage. Small amounts of vitamin A bring about good growth whereas considerably larger amounts are required for maximal blood levels and good liver storage.

In this connection it is of interest to mention that only one of four calves receiving the low intake of 32 units per kilogram body weight exhibited night blindness. We have previously demonstrated that the retinal requirement for vitamin A is very low and that with the ingestion of small amounts of vitamin A by rats, maximal concentrations of

vitamin A were obtained in the retinas even though the blood levels were low and the liver reserves were nil. It was therefore not surprising to find that three of the four calves receiving 32 units of vitamin A had normal dark adaptation despite the fact that the vitamin A concentration in the blood was very low. From a practical point of view, the blood level of vitamin A is a more sensitive indicator of the vitamin A status of the organism than is dark adaptation, as the latter becomes impaired only when the deficiency is of a more advanced degree.

SUMMARY AND CONCLUSIONS

Six groups of four calves each were fed various levels of vitamin A, ranging from 32 to 1024 U.S.P. units per kilogram of body weight per day. Data were obtained on rate of growth, blood levels of vitamin A and liver storage.

The results indicate that 32 units per kilogram of body weight just about cover the minimum requirement. At this level the calves grew fairly well and in most instances showed no evidences of night blindness. However, the blood concentration of vitamin A was low and there was little or no liver storage.

Maximum growth was obtained on an intake of 64 U.S.P. units per kilo of body weight.

The concentration of vitamin A in the blood was proportional to the intake until 512 units were given at which level maximal blood concentrations were obtained.

Liver storage of vitamin A was quite variable. The majority of the calves receiving 64 units per kilogram or below showed no liver storage. In general, liver stores were quite low for calves receiving 32, 64 and 128 units per kilogram, whereas moderate amounts of vitamin A were found in the livers of those fed 256 and 512 units and large amounts in the 1024 units group.

From the standpoint of both growth and liver storage the daily intake of vitamin A for young calves should be about 250 U.S.P. units per kilogram of body weight or 11,000 units per 100 pounds of liver weight.

The vitamin A requirements in calves are of the same order of magnitude as in young rats and in infants.

LITERATURE CITED

Boyer, P. D., P. H. Phillips, N. S. Lundquist, C. W. Jensen and I. W. Rupel Vitamin A and carotene requirements for the maintenance of adequate blood plasma vitamin A in the dairy calves J. Dairy Sci., vol. 25, p. 433.

GUILBERT, H. R., R. F. MILLER AND E. H. HUGHES 1937 The minimum vitamin A and carotene requirement of cattle, sheep and swine. J. Nutrition, vol. 13, p. 543.

LEWIS, J. M., AND CHARLES HAIG 1939 Vitamin A requirements in infancy as determined by dark adaptation. J. Pediat., vol. 15, p. 812.

LEWIS, J. M., O. BODANSKY, K. G. FALK AND G. MCGUIRE 1942 Vitamin A requirements in the rat. J. Nutrition, vol. 23, p. 351.

THE INFLUENCE OF ASCORBIC ACID ON THE ACTIVITY OF GONADOTROPIC HORMONES IN GUINEA PIGS [1]

J. T. REID AND J. F. SYKES

Departments of Dairy and Physiology and the Institute of Nutrition, Michigan State College, East Lansing

(Received for publication June 25, 1945)

Recent evidence has been presented which indicates that vitamin C is related to normal reproductive function.

In their studies on vitamin C-gonadotropic hormone relationships, Erb and Andrews ('42) found a 42 to 67% reduction in the plasma ascorbic acid concentration of dairy bulls within 24 hours after the injection of 2250 R. U. of gonadotropin. They also reported a 20 to 50% reduction in plasma vitamin C level of cows during the first 24-hour period following the injection of 1000 to 2250 R. U. of gonadotropic hormone. Further study by Andrews and Erb ('42) using the castrate bovine showed that gonadotropic hormone may act independently of the gonads in effecting a reduction of the plasma vitamin C level. These results indicated that vitamin C was in some manner concerned with the utilization of gonadotropic hormones.

Giedosz (cited by Di Cio and Schteingart, '42) however, showed that in rats the simultaneous injection with 50 mg. of vitamin C and 25 R. U. of pregnant mare serum gonadotropin did not effect a greater increase in the weight of testes, ovaries or uterus than did the injection of 25 R. U. of gonadotropin alone. In a further study Andrews and Erb ('43) found no relationship between the plasma ascorbic acid level and testes weight in young chicks, and concluded that the effects of gonadotropic hormone are not augmented by vitamin C.

Giedosz (cited by Di Cio and Schteingart, '42) however, showed that larger ovaries are produced in adult rabbits injected simultaneously with vitamin C and gonadotropin for 3 days than were produced in animals injected with hormone alone. He concluded that the hormone is more efficient when vitamin C saturation is accomplished. These findings were similar to those of Di Cio and Schteingart ('42) who used 2 to 4 months old white rats. While the latter investigators showed no

[1] Published as Journal Article no. 759 n. s., from the Michigan Agricultural Experiment Station.

appreciable increase in gonadal size of rats administered C alone over the normals, a much greater increase in the weight of gonads was found in those rats simultaneously injected with 50 mg. of vitamin C and 25 R. U. of gonadotropin than in those receiving gonadotropic hormone alone.

It seems possible to assume that the failure of Andrews et al. to potentiate the effects of gonadotropins with vitamin C in the rat and chicken was due to the fact that these animals are able to synthesize the vitamin. It was the object of this study to determine whether or not vitamin C increases the effects of gonadotropic hormone in the guinea pig since this animal does not synthesize the vitamin, but depends on a food source to supply its needs.

MATERIALS AND METHODS

Data on fifty-eight male and sixty-one female guinea pigs composing five groups were obtained in this study.

All groups received the basal, low vitamin C diet with certain modifications which will be discussed under each individual group. The basal ration had the following composition (parts by weight): skim milk (dried) 30, rolled oats 30, bran 30, butterfat 10, NaCl 1, and cod liver oil 1. The cod liver oil contained 400 and 3000 units per gram of vitamins D and A, respectively.

Group 1 received the low vitamin C diet throughout the experiment without supplementary vitamin C or gonadotropin. Group 2 received the basal diet and gonadotropin but did not receive supplementary ascorbic acid. After the appearance of scorbutic symptoms, gonadotropin was injected at two levels. One hundred R. U. and 50 R. U. of gonadotropin was injected daily into seventeen and twenty-five guinea pigs, respectively, for 4 days. Group 3 was depleted of vitamin C on the basal diet until deficiency symptoms were evident; and, then given 50 mg. of vitamin C daily for 3 days, followed by a 4-day period during which gonadotropin in addition to 50 mg. of ascorbic acid was administered. Twelve animals were injected with 100 R. U. of gonadotropin per day while the remaining twenty were injected with 50 R. U. per day. Group 4 received the basal diet supplemented with 2 mg. of ascorbic acid per guinea pig per day. When scorbutic symptoms were manifested in the animals of groups 1, 2 and 3, daily injections of 100 R. U. and 50 R. U. were administered, respectively, to eight and nine animals of group 4 for 4 days. Group 5 received the basal diet and 2 mg. of ascorbic acid per guinea pig per day until 9 days prior to the termination of the experiment when the daily diet for each animal was supplemented with 50

mg. of ascorbic acid. Six animals of this group were injected with 100 units of gonadotropin for 4 days while the remaining nine guinea pigs were injected with 50 units per animal for the same period of time.

In all cases where gonadotropin injections were given, the hormone was injected for 4 days and the animals were killed on the sixth day after the initial injection. The gonads were trimmed free of extraneous tissue, except for the epididymis of the testes, and weighed immediately. Gonadin serum from pregnant mares containing 50 R. U. per milliliter was used for the injections indicated. Supplementary vitamin C for the groups indicated above was mixed in the feed daily previous to feeding. The irritation effects which usually accompany injection of the vitamin were thus eliminated.

Since several workers, Rosenberg ('42), Farmer and Abt ('36) and Eddy and Dalldorf ('44), have indicated that the plasma vitamin C level reflects the degree of deficiency, this test as well as scorbutic symptoms was used as a criterion of the degree of deficiency. The vitamin C level was determined on the blood plasma of representative animals from each group. Care was taken in the handling of blood drawn from the heart for plasma ascorbic acid determinations to avoid exposure to light and heat. Although the general procedure outlined by Mindlin and Butler ('37) for the determination of vitamin C was employed, a modification (unpublished data) was introduced to preclude error due to a visually undetectable turbidity of the filtrates. This modification consisted of a complete reduction of the 2–6 dichlorophenol indophenol dye after reduction of the dye by the blank and unknown had been determined. A correction was then made for each on the basis that the completely reduced solution of dye and filtrate gave 100% transmission of light when the instrument was adjusted for 100% transmission with distilled water. Readings were made at 520 μ on a Cenco spectrophotelometer equipped with a Rubicon galvanometer.

RESULTS

The data in table 1 show the male and female gonad weights and the initial and terminal plasma vitamin C levels for groups 1, 2 and 3. Inasmuch as no difference between the effects of 100 or 50 units of gonadin was observed, the gonad weights for these animals have been grouped together.

The figures show that the average testes weight for the animals in groups 2 and 3 were, respectively, 1.81 and 1.83 times greater than those in group 1, whereas the average testes weight for group 3 was only 1.0094 times heavier than the male gonads in group 2.

The respective average ovary weights for groups 2 and 3 were 1.73 and 2.37 times as great as those in group 1, and the mean ovary weight for the animals of group 3 was 1.38 times as heavy as those in group 2. The differences observed between groups 1 and 2 may not be significant due to the small number of animals which survived in group 1. These data, however are an indication that gonadotropins are effective in the vitamin C deficient guinea pig and group 1 was included in the experimental set-up for this purpose.

TABLE 1

The effect of the low vitamin C diet, gonadotropin alone, and the combination of vitamin C and gonadotropin on gonad weight.

GROUP	SUPPLEMENT: Vitamin C	SUPPLEMENT: Gonado-tropin	MALES[1]: Mean gonad weight	FEMALES[1]: Mean gonad weight	PLASMA VITAMIN C[2]: Initial	PLASMA VITAMIN C[2]: Terminal	TERMINAL BODY WEIGHT
	mg.	*R.U.*	*gm.*	*mg.*	*mg. %*	*mg. %*	*gm.*
1	0	0	1.354 ± 0.20(10)	51 ± 8.15(3)[3]	0.258(6)	0.019(6)	326
2	0	50 or 100 (4 days)	2.455 ± 0.18(19)	88 ± 3.47(23)	0.318(18)	0.077(10)	312
3	50 (7 days)	50 or 100 (4 days)	2.478 ± 0.19(15)	121 ± 7.42(17)	0.304(18)	0.517(15)	371

[1] Figures in parentheses indicate number of animals or determinations.

[2] Average values.

[3] The small number of females in group 1 is actually the number that existed when the experiment terminated, and is small because of a high rate of mortality due to scurvy. Originally the number of animals in groups 1, 2 and 3 was comparable. Although the direction of the results was the same for each of the three females in group 1, the chief comparison to be stressed here is based on groups 2 and 3; i.e., demonstrating the potentiation of the effects of gonadotropins by vitamin C.

Although the supplementation of the diet of male animals with ascorbic acid did not potentiate the effects of gonadotropin, these data indicate a marked synergistic relationship involving the plasma vitamin C concentration and gonadotropic hormone in female guinea pigs as indicated by the increased gonad weights of the females in group 3 as compared to group 2.

The weight of the gonads and the initial and terminal plasma vitamin C values for groups 4 and 5 are shown in table 2. A comparison of these data with those for group 2 in table 1 shows that the average ovary weight of guinea pigs which were normal with respect to plasma ascorbic acid concentration and which were injected with gonadotropin was greater than in similar animals receiving the basal diet and gonadotropic hormone alone. The addition of 50 mg. of vitamin C daily for 9

days to normal guinea pigs did not increase the effectiveness of gonadotropins.

It will be noted that the terminal ascorbic acid values for group 4 are lower than the initial values and that in group 5 the addition of extra vitamin C did not produce terminal values comparable to those obtained in group 3. These, however, are average values and the lower terminal values are a result of the fact that 2 mg. of vitamin C per day in the ration failed to maintain the plasma ascorbic acid at comparable levels in all animals in these two groups.

TABLE 2

Effect of gonadotropin on guinea pigs with normal plasma vitamin C concentration.

GROUP	SUPPLEMENT Vitamin C	SUPPLEMENT Gonadotropin	MALES[1] Mean gonad weight	FEMALES[1] Mean gonad weight	PLASMA VITAMIN C[2] Initial	PLASMA VITAMIN C[2] Terminal	TERMINAL BODY WEIGHT
	mg.	*R.U.*	*gm.*	*mg.*	*mg. %*	*mg. %*	*gm.*
4	2 (daily)	50 or 100 (4 days)	2.134 ± 0.29(10)	134 ± 13.19(7)	0.234(9)	0.127(6)	420
5	2 (daily); 50 (9 days)	50 or 100 (4 days)	2.170 ± 0.57(4)	117 ± 7.02(11)	0.228(8)	0.326(6)	382

[1] Figures in parentheses indicate number of animals or determinations.

[2] Average values.

DISCUSSION

Inasmuch as the failure of other investigators to show an augmentation of the effects of gonadotropin by ascorbic acid may have been due to the use of experimental animals capable of ascorbic acid synthesis, the guinea pig was chosen as the test animal for this study by virtue of its inability to synthesize vitamin C. It seemed logical that the effects of the vitamin on gonadotropin utilization could be interpreted more conclusively in the guinea pig since this animal may be readily depleted or maintained at various vitamin C levels. In the first three groups receiving the low vitamin C diet, scorbutic symptoms accompanying a decreased plasma vitamin C concentration were manifested after a period of 3 to 4 weeks. Although a great response to the supplementary vitamin C was demonstrated by depleted females injected with gonadotropin, no noticeable potentiation was found in the males. This may be explained partially by a greater resistance to vitamin C depletion as evidenced by lack of marked scorbutic symptoms in the males as compared to the females.

The augmentation effects of ascorbic acid observed in female guinea pigs in this study offers support to the results obtained with rabbits and rats reported by Giedosz (cited by Di Cio and Schteingart, '42) and Di Cio and Schteingart ('42), respectively. The failure of Andrews and Erb ('43) to show the potentiation of gonadotropin effects by ascorbic acid in male chicks may be attributed to a sex as well as a species difference relative to the ascorbic acid-gonadotropin synergism. Almquist and Andrews ('43) also failed to duplicate the results of Di Cio and Schteingart ('42) with rats. It is difficult to offer an explanation for this discrepancy in results when the latter species was used.

The augmentation of the effects and the utilization of gonadotropic hormone produced by vitamin C appear to vary with sex as well as species. The difficulty commonly encountered in the treatment of sterility in various animals with gonadotropins might be attributed in part to this fact.

SUMMARY

Gonadotropin injections in vitamin C depleted females resulted in an average ovary weight 1.73 times greater than in depleted females not receiving gonadotropin injections. The addition of vitamin C further increased the gonadotropic effects and produced ovaries 1.38 times heavier than in those receiving gonadotropin alone.

The injections of 50 or 100 R.U. of gonadotropin into vitamin C depleted male guinea pigs increased the testis weight 1.81 times as compared to the depleted guinea pigs not receiving gonadotropin. Supplementing the basal diet with 50 mg. of vitamin C per day, however, did not further increase the testis weight produced by gonadotropin alone.

The augmentation of the effects and the utilization of gonadotropic hormone caused by vitamin C appears to differ with sex as well as species.

LITERATURE CITED

ALMQUIST, J. O., AND F. N. ANDREWS 1943 Ascorbic acid-gonadotropic hormone relationships. J. Animal Sci., vol. 2, p. 356.

ANDREWS, F. N., AND R. E. ERB 1942 Effect of the gonadotropic substance of pregnant mare's serum on the blood plasma-ascorbic acid of the castrate bovine. Endocrinol., vol. 30, p. 671.

——— 1943 Ascorbic acid-gonadotropic hormone relationships in the chick. Endocrinol., vol. 32, p. 140.

DI CIO, A. V., AND M. SCHTEINGART 1942 The influence of ascorbic acid on the activity of gonadotropic hormones. Endocrinol., vol. 30, p. 263.

EDDY, W. H., AND G. DALLDORF 1944 The Avitaminoses. Williams and Wilkins Company, Baltimore, 3rd ed., pp. 294–296.

ERB, R. E., AND F. N. ANDREWS 1942 Effect of the gonadotropic substance of pregnant mare's serum on the blood plasma-ascorbic acid of the bovine. Endocrinol., vol. 30, p. 258.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood Proc. Soc. Exp. Biol. Med., vol. 34, p. 146.

MINDLIN, R. L., AND A. M. BUTLER 1937 The determination of ascorbic acid in plasma; a macromethod and micromethod. J. Biol. Chem., vol. 122, p. 673.

ROSENBERG, H. R. 1942 Chemistry and Physiology of the Vitamins. Interscience Publishers, Inc., New York, p. 333.

THE AVAILABILITY OF THE IRON IN HAWAIIAN-GROWN VEGETABLES [1]

CAREY D. MILLER AND LUCILLE LOUIS [2]
Nutrition Department, Hawaii Agricultural Experiment Station,
University of Hawaii, Honolulu, T. H.

ONE FIGURE

(Received for publication July 9, 1945)

In Hawaii there has been for some years a popular belief that locally produced vegetables may be low in minerals, notably iron, and that people residing in the Islands tend to become anemic. These erroneous ideas probably arose because it is generally known that pineapples grown in certain areas become chlorotic and must be sprayed with iron sulfate. Johnson ('24) showed that precipitation of the iron by manganese in certain soils deprives the plant of the iron required for chlorophyll synthesis despite the presence of abundant iron. Later work (Sideris, Young and Krauss, '43) has proved that not only manganese, but calcium, phosphorus and the pH of the soil play important roles in the utilization of iron by the pineapple plant. Other plants growing in the same soil with chlorotic pineapple plants do not show chlorosis.

Blood studies (Hamre, '39) on local residents have shown the usual range of hemoglobin and red cell counts found on the mainland. Analyses of a large number of locally produced vegetables for calcium, phosphorus and iron (to be published elsewhere) have shown no greater range in values than reported for other regions.

This study reports the results of iron analyses of fifteen vegetables and two seaweeds grown in Hawaii and the availability of the iron for the regeneration of hemoglobin in anemic rats.

EXPERIMENTAL

Rats. Young rats were made anemic by a modification of the Elvehjem and Kemmerer ('31) technique. The mothers were fed the stock diet throughout the lactation period; but from the time the young were

[1] Published with the approval of the director as Technical Paper no. 127 of the Hawaii Agricultural Experiment Station.

[2] Resigned February, 1942.

14 days old, the mothers were removed from the cages for feeding twice a day and their coats brushed before returning them to the cages with the young. At 17 days of age the young and the mothers had access to whole powdered milk. The young were weaned at 21 days, placed in individual, new, rust-free, galvanized cages, and fed an exclusive diet of whole milk powder and tap water.

As Smith and Otis ('37) had reported a greater hemoglobin response for females than males when fed equivalent amounts of iron, only male rats were used in our experiments. Weekly blood samples were taken from the rats' tails by snipping off a bit of the tip or by cutting a small gash near the end of the tail with a sharp razor. Care was taken to obtain a full-flowing sample and to stop the bleeding promptly after the samples were taken. The average period for depletion of hemoglobin stores was 26 days. Most of the rats were depleted to a level of about 3.4 gm. of hemoglobin per 100 ml. of blood in 21 to 24 days but two litters for undetermined reasons took 30 and 40 days.

To determine a curve of response, one hundred male rats were divided into nine groups, with proper distribution of the rats from different litters to each group, and, after depletion of hemoglobin, were fed daily (except Sunday) supplements of iron (ferric chloride) for a period of 6 weeks as follows: 0.015, 0.03, 0.06, 0.10, 0.13, 0.17, 0.20 and 0.25 mg. Each rat was also fed daily supplements of 0.05 mg. copper in the form of a dilute solution of $CuSO_4$ and 0.04 mg. manganese in the form of a dilute $MnSO_4$ solution. One group of eleven rats received these supplements without iron. Statistical analysis of the data on gains in hemoglobin showed that feeding the supplements for 4 weeks produced as significant results as for 5 or 6 weeks, somewhat better than 3 weeks and definitely better than 2 weeks. The minimum number of rats necessary to give a significant result in hemoglobin response after 4 weeks of feeding was calculated to be nine for the procedure and the rat colony used. A linear regression curve was calculated from the data for the 4-week feeding period and used as a curve of reference for the experiments which followed (fig. 1). Complete data for all groups of rats used for the curve of response have been omitted from series 1, table 1, in order to save space and only the data for the groups fed supplements of copper and manganese without iron, 0.06 mg. of iron, and taro leaves are included.

For the final tests of the available iron in foods, two groups of rats in each series received 0.06 and 0.08 mg. of iron. All groups received the copper and manganese supplements. The plan was to feed all supplements for a period of 4 weeks. It was necessary to modify the plan

of the feeding experiments when, in March, 1942, it was obvious that our supplies of powdered whole milk were running low and all stores of such foods in Hawaii were being reserved for human consumption. There were, moreover, shortages in staff and the uncertainty as to how long research work could continue. Consequently, in order to complete the project and utilize the rats on the experiment, the period for feeding the supplements for series 5 and 6 was reduced to 3 weeks (see fig. 1 for linear regression curve) and for series 6 only one group was fed 0.06 mg. of iron as a positive control.

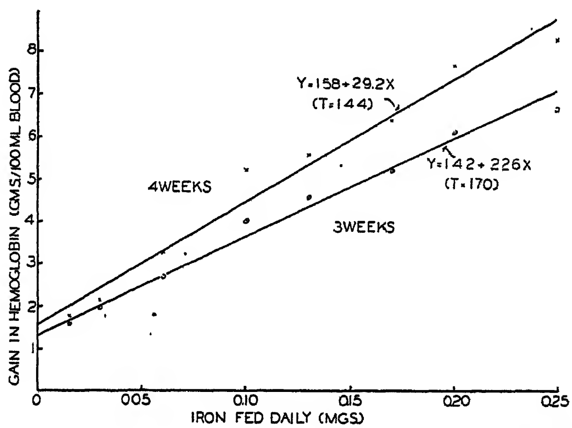


Fig. 1 Curves of response (linear regression curves for gains in grams of hemoglobin per 100 ml. of blood in relation to milligrams of iron fed daily, with supplements of copper and manganese, for 3– and 4–week periods).

Supplements and their preparation. All of the vegetables were fed in the fresh or cooked state as it was thought that drying and grinding might adversely affect the utilization of the iron. The limu (Hawaiian name for all seaweeds), gathered from the seashore or rocks, were thoroughly cleaned and washed and fed in the raw state. The vegetables were prepared as for human consumption, discarding such portions as old leaves, over mature beans, or lower tough stalks of asparagus. In the case of the white mustard cabbage, a portion of the large white petiole was discarded in order that a smaller sample with a higher iron content might be used for feeding purposes. (The entire edible portion of the plant contained 0.00053% of iron by one set of analyses, whereas the composite sample used for rat feeding contained 0.00156% of iron.)

The vegetables were cooked in aluminum vessels and chopped on a rubber chopping board with a stainless steel knife. Samples were prepared once or twice a week and stored in glass jars in the refrigerator until fed.

The green leafy vegetables, green beans, and cowpeas were steamed for 10 to 15 minutes, without loss of liquid. The green soy beans were cooked in boiling water in the pods for 20 minutes; the beans were shelled from the pods and chopped before feeding. Other vegetables — carrots, asparagus, green lima beans (shelled) — were cooked for varying lengths of time until tender, without loss of liquid. The cooked, ground taro was a commercial product locally known as paiai, containing about 30% solids. It was put through a coarse cloth (poi cloth) without the addition of water to remove the lumps and to make a more uniform sample.

The quantities of the supplements fed were based on previous iron analyses of local vegetables made in the department laboratory or a special preliminary analysis. The final analyses (table 1) were made on composite samples made up of aliquots from each lot of vegetables, cooked, chopped and prepared for rat feeding. These samples were stored in glass jars at a temperature of approximately 34°F. until analyzed. Measured, small quantities of a 10% formalin solution were added to the jars as an added precaution against molds. The formalin solution was analyzed for iron and found to contain negligible amounts.

The aim was to feed a quantity of the food that would furnish approximately 0.06 to 0.08 mg. of iron in the daily supplements. In a number of cases the quantity of iron in the composite sample, proved to be either higher or lower than that found by preliminary analyses, so that the amount of iron furnished in the sample fed proved to be smaller (limu lipeepee) or larger (white mustard and green mustard cabbage) than was calculated.

Iron analyses. In preparing the samples for analyses, every precaution was taken to guard against contamination with iron from outside sources. The samples were ashed without drying. For each sample analyzed, two checks were run for the entire process from ashing to colorimetric readings — one a complete blank, the other with a known quantity of added iron. The Saywell and Cunningham ('37) method of color development with ortho-phenanthroline was followed. A Klett-Summerson photoelectric colorimeter was used for reading the solutions.

The iron solutions for feeding were made from either special reagent ferric chloride or pure iron wire, and the iron contents were checked by the ortho-phenanthroline method. The copper and manganese salts were tested for their iron contents and found to have only a trace — too small an amount to detect in the dilute solutions used.

Hemoglobin determinations. Hemoglobin was determined by the acid hematin method, using a Klett-Summerson photoelectric colori-

TABLE 1

Summary of the results of feeding various Hawaiian foods and ferric chloride as sources of iron to anemic rats.

SUPPLEMENTS FED DAILY (EXCEPT SUNDAYS)	IRON IN FOOD		NO. OF RATS	HEMOGLOBIN			APPROXIMATE % OF FE UTILIZED	AVERAGE WEIGHTS		
	mg./100 gm.	Furnished daily		When started	4th week Level	4th week Gain		Initial	At depletion	End of expt.
		mg.		*gm./100 ml.*	*gm./100 ml.*	*gm./100 ml.*	%	*gm.*	*gm.*	*gm.*
Series 1.										
$CuSO_4$(0.05 mg. Cu) + $MnSO_4$(0.04 mg. Mn)		0	11	3.3	4.5	1.2		42	93	146
Ferric chloride		0.06	11	3.2	6.5	3.3		41	90	180
Taro leaves 5 gm.	1.42	0.071	10	3.4	6.9	3.5	93	45	104	196
Series 2.										
Ferric chloride		0.06	10	3.4	7.2	3.8		43	98	183
Ferric chloride		0.08	10	3.5	7.7	4.2		43	96	187
Cowpeas (fresh pods) 8 gm.	1.12	0.09	10	3.4	7.0	3.6	65	44	95	195
Swamp cabbage 5 gm.	2.62	0.131	10	3.4	7.7	4.3	59	44	98	183
Green beans 10 gm.	0.78	0.078	10	3.5	6.4	2.9	49	44	99	181
Series 3.										
Ferric chloride		0.06	10	3.3	6.6	3.3		39	87	178
Ferric chloride		0.08	10	3.3	7.3	4.0		39	88	180
Amaranth 5 gm.	2.97	0.149	10	3.3	6.2	2.9	32	40	87	177
Lima beans (green) 4 gm.	1.83	0.073	10	3.2	6.3	3.1	74	39	80	185
Watercress 7 gm.	1.37	0.096	10	3.2	5.6	2.4	31	38	81	174
Series 4.										
Ferric chloride		0.06	10	3.6	7.0	3.4		41	93	193
Ferric chloride		0.08	10	3.7	7.3	3.6		42	97	193
White mustard cabbage 9 gm.	1.56	0.14	10	3.6	6.5	2.9	34	41	95	200
Green mustard cabbage 9 gm.	1.21	0.109	10	3.6	5.4	1.8	10	40	95	179
Paiai (taro corm) 7 gm.	1.52	0.106	10	3.6	7.9	4.3	93	40	98	200
Series 5.					Third week					
Ferric chloride		0.06	10	3.6	6.1	2.5		43	99	166
Ferric chloride		0.08	10	3.7	6.7	3.0		44	100	171
Asparagus 10 gm.	1.82	0.182	10	3.7	5.3	1.6	6	42	98	163
Broccoli 10 gm.	1.02	0.102	10	3.6	6.0	2.4	52	43	100	180
Carrots 10 gm.	0.47	0.047	10	3.6	5.1	1.5	17	42	100	171
Series 6.										
Ferric chloride		0.06	10	3.7	6.5	2.8		44	105	165
Green soybeans 3.5 gm.	2.98	0.104	10	3.6	7.4	3.8	96	44	105	182
Limu lipoa 1.0 gm.	15.02	0.15	10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

meter for measurement of the concentration of the solutions. The readings of the colorimeter were standardized with a series of acid hematin solutions made from a sample of blood of which the iron content was determined by the Wong ('28) method.

"Available" or acid-soluble iron. Many unsuccessful attempts to determine available iron were made in this laboratory using the alpha alpha-dipyridyl method and its numerous modifications (Shackleton and McCance, '36; Kohler, Elvehjem and Hart, '36; Ranganathan, '38.) We obtained more consistent results if the acetic acid solutions were evaporated and ashed and the color developed with ortho-phenanthroline than if dipyridyl was used for development of color directly in the extracts. For all but one of the foods studied, acid extracts were also made with 7.5% sulphuric acid.

Briefly the methods used were as follows: Raw or cooked food samples were ground in a special aluminum grinder. For the acetic acid extracts, 40 to 50 ml. of 10% acetic acid were added to 20 to 25 gm. of finely ground samples and the mixtures heated in beakers on the waterbath for 2 to 3 hours. After adding 1 ml. of 10% hydroxylamine hydrochloride, the heating was continued for another hour. After standing overnight, the samples were transferred to 50 ml. tubes and centrifuged. The supernatant liquid was filtered off and the residue was washed four times with 4% acetic acid followed by centrifuging. The solutions were transferred to silica dishes and after evaporation on a waterbath residues were ashed as for total iron with the usual precautions and customary blanks.

For the sulphuric acid extracts, 20 to 30 ml. of 7.5% sulphuric acid were added to samples weighing 10 to 15 gm. The mixtures were heated on the waterbath and centrifuged in the same way as for the acetic acid extractions. After evaporating nearly to dryness, digestion was completed by the addition of nitric and perchloric acids. Solutions were made to volume and iron determined as previously described.

RESULTS AND DISCUSSION

The Hawaiian-grown foods studied, their total iron contents, and their hemoglobin regenerating values as determined by bioassay are summarized in table 1.

The values for total iron in six of the vegetables studied were compared with values in the literature that were obtained when special precautions were taken to guard against iron contamination (Stiebling, '32; Peterson and Elvehjem, '28). Three of the Hawaiian-grown vegetables — asparagus, green snap beans and lima beans — had as much iron

as the mainland products, and two — broccoli and carrots — had slightly less iron. The Hawaiian-grown watercress (composed of 3 to 4 inches of stems plus leaves) contained much less iron than was reported by these two laboratories. However, our results compare favorably with those reported for the iron content of cultivated watercress from West Virginia (Stegner, '44). The green soybeans used contained as much iron as those grown in Illinois (Woodruff and Klaas, '38). Reliable iron figures for the total iron content of the other vegetables studied could not be found in the literature.

The percentages of "available iron" determined by the two acid extraction methods were in most cases very divergent and neither of them showed any correlation with the results by bioassay. The data are summarized in table 2.

TABLE 2

"Availability" of iron. (Expressed as percentage of total iron.)

FOOD	BIOASSAY	SOLUBLE IN 10% ACETIC ACID	SOLUBLE IN 7.5% SULPHURIC ACID	FOOD	BIOASSAY	SOLUBLE IN 10% ACETIC ACID	SOLUBLE IN 7.5% SULPHURIC ACID
	%	%	%		%	%	%
Amaranth	32	14	99	Cabbage			
Asparagus	6	26	86	green mustard	10	47	74
				swamp	59	4	69
Beans				white mustard	34	33	103
lima (green)	74	10	101	Limu lipoa			
green snap	49	31	95	(seaweed)	62	23	99
green soy	96	12	(113)				
Belembe	31	5	92	Paiai (taro corm)	93	49	92
Broccoli	52	29	99	Taro leaves	93	4	..
Cowpeas (pods)	65	13	99	Watercress	31	21	81
Carrots	17	45	(115)				

On the basis of bioassays with anemic rats, nine of the foods studied had 50% or more of the iron in available form and seven had less than 50%. In agreement with other published reports (Sheets and Ward, '40; Theriault and Fellers, '42), we found fresh legume seeds to have a relatively high percentage of available iron — soybeans 96% and fresh lima beans 74%. The green podded legumes with seeds — cowpeas and green snap beans — yielded lower values, 65 and 49%, respectively.

The iron in taro corms (paiai) and taro leaves was found to be 93% available. Both foods are important items in the diet of ancient and modern Polynesians.

Others have reported the iron of green leafy vegetables to be generally less available than the iron in legumes (Sheets and Ward, '40; Theriault and Fellers, '42). We found the available iron by bioassay to vary greatly — from 93% in taro leaves to 10% in green mustard cabbage. Two leafy vegetables, taro leaves and swamp cabbage, had more than 50% of the iron available, four — white mustard cabbage, amaranth, watercress and belembe — had about 30% available and one, green mustard cabbage, had only 10% available.

We found broccoli to have only 52% of its iron available by bioassay, whereas Hastings, Fellers and Fitzgerald ('41) reported 71% for raw broccoli and 94% for fresh frozen broccoli. Fresh, cooked, Hawaiian-grown asparagus yielded the lowest value for available iron of all the vegetables studied — 6%. Theriault and Fellers ('42) report 55% available iron bioassay for quick-frozen asparagus fed in dried form.

Insufficient iron was furnished by one of the seaweeds (limu lipeepee, a species of Laurencia) to make an estimate of its availability. The quantity fed was based on a preliminary iron analysis of a sample that was probably not fully freed from sand. The data in table 1 for the other seaweed, limu lipoa, indicate that the iron is relatively well utilized.

On the basis of these experiments, eight vegetables and one seaweed appear to be good sources of iron for hemoglobin building. A number of the green leafy vegetables commonly considered good sources of iron were found to be relatively poor for hemoglobin regeneration in the anemic rat.

The large gains in weight (table 1) made by our rats during the 3- or 4-week period when either inorganic iron or the food supplements were added to the milk powder diet appear to be greater than are reported by other investigators (Harris, Mosher and Bunker, '39; Nakamura and Mitchell, '43). A portion of the iron ingested was undoubtedly used for purposes other than blood hemoglobin as Hahn et al. ('39) have pointed out that iron exists in many places in the body other than red cell hemoglobin. If growth had been retarded by restriction of the basal diet of milk powder, probably the increases in hemoglobin would have been greater, but this should not have influenced the relationship of the increases between the groups fed food supplements and the controls.

Sherman ('41) has questioned whether the availability of iron of foods based on studies with anemic rats has any significance for humans. However, there would seem to be just as much justification for accepting the comparative values for the utilization of iron in foods as for accepting the comparative vitamin values of foods (especially vitamin A and carotene) and biological values of proteins determined by means of

rat experiments. All such values are relative and merely guides in planning human dietaries. Moreover, since Hahn et al. ('39) have shown by means of radioactive iron studies that the anemic animal absorbs iron more readily than the nonanemic one, it would appear unlikely that the iron of foods poorly used by anemic rats would be better utilized by normal humans.

SUMMARY AND CONCLUSIONS

The availability of the iron in fifteen Hawaiian-grown vegetables and one seaweed was determined by bioassay.

The mean values for hemoglobin regeneration in groups of anemic rats fed varying amounts of the foods for periods of 3 or 4 weeks were compared with those of control groups fed pure ferric chloride. All rats were given supplements of copper and manganese.

In comparison with the utilization of the iron in ferric chloride, the percentages of the total iron available in the foods studied were found to be as follows: green soybeans 96, taro corms 93, taro leaves 93, fresh lima beans 74, cowpeas (pods) 65, a seaweed, limu lipoa (Haliseris plagiogramma) 62, swamp cabbage (Ipomoea aquatica) 59, broccoli 52, green snap beans 49, white mustard cabbage (Brassica chinensis) 34, amaranth (Amaranthus gangeticus) 32, watercress 31, belembe (Xathosoma brasiliense) 31, carrots 17, green mustard cabbage (Brassica juncea) 10, and asparagus 6.

The superior quality of the iron of legume seeds for hemoglobin regeneration in the rat was confirmed. Taro corms and taro leaves, both important items in the diet of the Polynesians, were shown to have a high percentage of available iron.

The percentage of the food iron soluble in dilute acetic or in dilute sulphuric acid bore no relationship to the availability as determined by bioassay.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Donald L. Van Horn for assistance with a portion of the statistical analysis and Kisako Yanazawa and Mary Nagai for care of the experimental animals.

LITERATURE CITED

ELVEHJEM, C. A., AND A. R. KEMMERER 1931 An improved technique for the production of nutritional anemia in rats. J. Biol. Chem., vol. 93, p. 189.

HAHN, P. F., W. F. BALE, E. O. LAWRENCE AND G. H. WHIPPLE 1939 Radioactive iron and its metabolism in anemia. J. Exp. Med., vol. 69, p. 739.

HAMRE, C. J. 1939 A blood survey of 137 young men and 112 young women. Trans. 49th Annual Meeting, Hawaii Terr. Med. Assn., p. 98.

HARRIS, R. S., L. M. MOSHER AND J. W. M. BUNKER 1939 The nutritional availability of iron in molasses. Am. J. Digest. Dis., vol. 6, p. 459.

HASTINGS, W. H., C. R. FELLERS AND G. A. FITZGERALD 1941 Effect of freezing on the available iron content of foods. Proc. Am. Inst. of Refrig., p. 1.

JOHNSON, M. O. 1924 Manganese chlorosis of pineapples; its cause and control. Hawaii Agr. Exp. Sta. Bull. 52.

KOHLER, G .O., C. A. ELVEHJEM AND E. B. HART 1936 Modifications of the bipyridine method for available iron. J. Biol. Chem., vol. 113, p. 49.

NAKAMURA, F. I., AND H. H. MITCHELL 1943 The utilization for hemoglobin regeneration of the iron in salts used in the enrichment of flour and bread. J. Nutrition, vol. 25, p. 39.

PETERSON, W. H., AND C. A. ELVEHJEM 1928 The iron content of plant and animal foods. J. Biol. Chem., vol. 78, p. 215.

RANGANATHAN, S. 1938 The "available" iron in some common Indian foodstuffs, determined by the alpha alpha-dipyridine method. Indian J. of Med. Res., vol. 25, p. 677.

SAYWELL, L. G., AND B. B. CUNNINGHAM 1937 Determination of iron — colorimetric *o*-phenanthroline method. Ind. Eng. Chem. Anal. Ed., vol. 9, p. 67.

SHACKLETON, L., AND R. A. MCCANCE 1936 The ionizable iron in foods. Biochem. J., vol. 30, p. 582.

SHEETS, O., AND M. V. WARD 1940 Studies in nutritional anemia. Miss. Agr. Exp. Sta. Tech. Bull. 26.

SHERMAN, H. C. 1941 Chemistry of Food and Nutrition. Macmillan, New York.

SIDERIS, C. P., H. Y. YOUNG AND B. H. KRAUSS 1943 Effect of iron on the growth and ash constituents of Ananas comosus (L.) Merr. Plant Physiol., vol. 18, p. 608.

SMITH, M. C., AND L. OTIS 1937 Hemoglobin regeneration in anemic rats in relation to iron intake. J. Nutrition, vol. 13, p. 573.

STEGNER, M. 1944 Home Ec. Consultant, New York. Personal communication.

STIEBLING, H. K. 1932 The iron content of vegetables and fruits. U.S.D.A. Cir. 205.

THERIAULT, F. R., AND C. R. FELLERS 1942 Effect of freezing and of canning in glass and in tin on available iron content of foods. Food Research, vol. 7, p. 503.

WONG, S. Y. 1928 Colorimetric determination of iron and hemoglobin in blood. J. Biol. Chem, vol. 77, p. 409.

WOODRUFF, S., AND H. KLASS 1938 A study of soybean varieties with reference to their use as food. Univ. Ill. Agr. Exp. Sta. Bull. 443.

BORDEN AWARD IN NUTRITION

The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1946. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

W. E. KRAUSS
Ohio Agricultural Experiment Station
Wooster, Ohio

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1946 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1946.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1945 must be in the hands of the Secretary by January 10, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

H. E. CARTER
Noyes Laboratory of Chemistry
University of Illinois
Urbana, Illinois

SECRETARY, AMERICAN INSTITUTE OF NUTRITION

INDEX